EFFECTS OF SWIMMING AND CREATINE SUPPLEMENTATION ON CULTURED FISHES

A Dissertation

by

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ABSTRACT

Improvements in production efficiency of aquaculture are necessary to provide seafood to feed a growing human population. Therefore, a series of studies were conducted to evaluate two different means of improving growth and production efficiency of several fish species.

The first study evaluated forced swimming as a means to improve growth of hybrid striped bass, red drum, and tilapia. Each species was subjected to a water current of 1 body length per second (bl/s) and compared to controls in static water for 7 weeks. Results indicated that continuous swimming beneficially affected aspects of tilapia and red drum growth and altered their body composition. Reduced growth was observed in hybrid striped subjected to flowing water, possibly due to consistently wary feeding behavior.

A second study, consisting of seven separate comparative feeding trials evaluated dietary creatine on channel catfish, hybrid striped bass and red drum under different salinity conditions which could influence the energetics of osmoregulation. In channel catfish fry, growth performance improved with supplemental creatine (2% of diet) over an 8-week feeding period at 8 ppt salinity. In another trial, there were no discernable differences in growth performance of catfish fry during a freshwater (2 ppt) phase, but there were significant improvements in survival of catfish in the brackish water (10 ppt) phase. In both trials, muscle creatine levels significantly increased with dietary supplementation.

Another 12-week, two-part feeding trial was conducted to evaluate dietary creatine supplementation on hybrid striped bass under different water salinity. During the low salinity (5 ppt) phase, there were no discernable differences in growth performance of juvenile hybrid

striped bass. However, significant improvements in weight gain were found in fish fed the diets supplemented with 2% and 4% creatine during the brackish-water (15 ppt) phase.

To evaluate dietary creatine supplementation in red drum, four separate feeding trials were conducted at various salinities. Significant improvements in growth performance were detected in juvenile red drum in all trials. A minimum creatine supplementation of 1.98% of diet provided optimal weight gain of red drum. Thus, this dissertation identified two different means of improving growth efficiency of three different fish species.

DEDICATION

To those whose name I share.

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CHAPTER 1

INTRODUCTION

Aquaculture is the fastest growing agriculture industry, providing high-quality protein to a growing human population (Fisheries 2011, Naylor *et al.* 2000). To allow further development of aquaculture, new strategies must be incorporated to improve production efficiency and health of cultured aquatic species. There are many avenues for potential improvement such as genetic advancements (Saura *et al.* 2016, Khaw *et al.* 2016) through selective breeding and broodstock management (Rhody *et al.* 2014), and improved culture system designs for water quality enhancement (Brown *et al.* 2012, Ramanan *et al.* 2010) to name a few. Two significant factors that may direct impact production efficiency and costs are the development of innovative culturing practices and enhancing fish performance with less expensive feeds.

Forced Swimming

Aquaculture is somewhat unique to animal production due to the level of environmental control the culturist can impose on a large number of animals. In the recent past, several reports of forcing fish swim under continuous low speeds [(1 body length/s (bl/s)] has resulted in them outperforming fish raised in a typical static culture system such as ponds, cages, or tanks (Grisdale-Helland *et al.* 2013, Palstra and Planas 2011, Kieffer 2010). Interest in applying low speed aerobic exercise to cultured fish has grown, particularly in species with active life history such as salmonids (Davison 1997, Grisdale-Helland *et al.* 2013, Palstra and Planas 2011, Velez *et al.* 2017). Yet, the evaluation of continuous swimming at low speeds on growth performance of representative species produced in aquaculture including red drum (*Sciaenops ocellatus*),

tilapia (*Oreochromis niloticus*), and hybrid striped bass (*Morone saxatilis*) is lacking. Although elevated activity increases metabolic demand, exercise has been shown in some species to improve robustness, social behavior, growth performance, immune responses, and carbohydrate utilization.

Exercise has been shown to affect many aspects of teleost physiology. Growth performance is enhanced due to muscle hypertrophy as well as hyperplasia. Furthermore, red muscle percentage, in respect to total muscle percentage, increases, and white muscle tissue is far less affected (Davison 1997). Consequently, exercised fish often favor lipid oxidation (Davison 1997). This is evident when muscle lipid concentration is reduced in exercised fish trained in fast current, whilst having minimum impact on glycogen stores (Davison and Goldspink 1977). Generally, exercised fish have been shown to have smaller livers and increased glycogen stores in muscle tissue (Davison 1989, Karlsen *et al.* 2006, Choi and Weber 2016).

Producing anabolic hormones, in particular insulin-like growth factor (IGF-1), and the reduction of catecholamines is a predominate focus in improving farm animal performance (Hossner 2005, Mommsen and Moon 2001, Velez *et al.* 2017). This is because IGF-1 positively correlates with growth in fishes (Beckman 2011), and aerobic exercise leads to the increase of serum IGF-1 and the upregulation of IGF-1 expression in the liver of gilthead sea bream (Mommsen and Moon 2001, Sanchez-Gurmaches *et al.* 2013, Velez *et al.* 2015). Exercise also leads to the reduction of catabolic enzymes such as adrenalin and cortisol (Woodward and Smith 1985, Davison 1997); reducing the negative effects of stressors such as hauling (Arbelaez-Rojas *et al.* 2013) or crowding (Young and Cech Jr 1993) of fish. Hierarchal dominance is a common stressor leading to a reduction in growth performance of aggressive fishes (Ejike and Schreck 1980, Alanara 1996, Morandini *et al.* 2014). Exercise reduces incidents of hierarchy behavior

leading to improved growth rates and less fin damage (Jobling *et al.* 1993, Adams *et al.* 1995, Jorgensen and Jobling 1993).

In addition to modifying specific characteristics of culture systems to alter fish behavior and thereby improve their growth and production efficiency, nutrition is another factor that can dramatically influence the cost effectiveness of aquacultural production. There has been some evidence indicating carnivorous salmonid species utilize carbohydrates more efficiently under sustained swimming (Kieffer *et al.* 1998). Red drum is a carnivorous species raised in the southern region of the United States, Mexico, Ecuador, and China. It is fast-growing species cultured for both food and stock enhancement. Improved utilization of carbohydrates by the carnivorous red drum may be another potential benefit of imposing continuous activity as seen in salmonid species (Kieffer *et al.* 1998). Manipulation of diet composition including the supplementation of certain nutrients involved in energy metabolism such as creatine also may influence fish growth and production efficiency.

Creatine

Creatine is currently classified as a non-essential organic acid commonly found in the flesh of animals. Anabolized from arginine, glycine, and methylated by methionine, creatine synthesis begins in the kidney and ends in the liver. The majority of creatine is taken up by the muscle tissue which holds approximately 95% of total creatine found in animals. Humans turn over roughly 2 g of creatine/day, which is replenished by the consumption of flesh from animals as well as by endogenous synthesis.

Creatine primarily functions as a regenerative energy source with storage of phosphorus as creatine phosphate (CrP) for energy metabolism. CrP is anabolized by an iso-form of creatine

kinase (Mi-CK) and is stored as phosphocreatine in muscle tissue. In times of intense energy expenditure, another iso-form of creatine kinase (MM-CK) or (MB-CK) liberates phosphorus from creatine and attaches the free phosphate to ADP; therefore, CrP serves as a reservoir of phosphate to replenish energy (ATP) according to the following reactions (Wyss and Kaddurah-Daouk 2000).

$$ATP + Cr \leftrightarrow ADP + CrP$$

There are several other functions of creatine that have been documented: it serves as a cellular buffer (Newsholme and Beis 1996), improves neuromuscular control (Iqbal *et al.* 2015), and increases protein synthesis (Shankaran *et al.* 2016, Kreider *et al.* 1998). Inability to endogenously produce sufficient creatine in humans leads to mild or severe intellectual inabilities (Hanna-El-Daher and Braissant 2016). Thus, it is well established that creatine is necessary for normal animal development, health and survival. However, whether dietary supplementation of creatine can improve some aspects of growth and metabolism has not been adequately addressed in cultured fish.

Menhaden (*Brevoortia patronus*), anchovy (*Engraulis encrasicolus*) and sardines (*Sardina pilchardus*) are small pelagic species which are collected by capture fisheries and used to produce fishmeal which has been a prominent feedstuff included in the diet of many aquacultured organisms (Naylor *et al.* 2000). Fishmeal is high in protein with an excellent amino acid profile, but due to increasing demand and limited supply, the cost of fishmeal has increased dramatically over the last few decades such that now it is primarily incorporated in diets of carnivorous species to provide optimal growth and wellbeing. Fishmeal also provides conditionally essential nutrients such as creatine and taurine which are largely absent in plant feedstuffs. Thus, as fishmeal in the diet of various aquatic species is replaced by less expensive

plant feedstuffs, the essentiality of certain compounds such as taurine to carnivorous fish species has become evident in recent years. A similar situation may be associated with creatine which also is devoid in plant feedstuffs that are increasingly being used to replace fishmeal and other meat products in aquafeeds.

Creatine supplementation to the diet affects growth, immunity, and ergogenic responses in mammals (Volek and Rawson 2004, O'Quinn *et al.* 2000). In fish, dietary creatine supplementation has been shown to affect sprint endurance (McFarlane *et al.* 2001) and osmoregulation (Weng *et al.* 2002). However, very limited research has addressed the effects of creatine on growth. This is likely because creatine is considered to be a non-essential organic acid that is not typically supplemented in diet formulations. As mentioned earlier, there is interest in reducing the inclusion of fishmeal and other animal protein meals in aquafeeds with less expensive plant protein feedstuffs, but plant proteins are devoid of creatine. Though considered non-essential, creatine may be conditionally essential for some species. Therefore, supplementation of creatine in plant-based diets may be required for the targeted species to exhibit comparable growth, survival, and feed efficiency as when fed animal-based diets. Therefore, this project evaluated creatine, a popular oral non-protein bound organic acid, in the diet of channel catfish, red drum and hybrid striped bass.

CHAPTER II

EFFECTS OF SUSTAINED SWIMMING EXERCISE ON GROWTH RESPONSES OF NILE TILAPIA, RED DRUM, AND HYBRID STRIPED BASS

II.1 Introduction

Subjecting fish to exercise has been shown to increase growth of many salmonid species; however, very limited research has investigated the effects of exercise on growth of warm-water species such as cichlids and sciaenids. This is likely because sustained swimming is not as ostensibly a part of the natural activity of those species compared to salmonids, and no cost-effective way to exercise these species under an aquaculture setting has been devised. However, with the advancement of recirculating aquaculture systems, in-pond raceways, and flow-through systems which continuously move water, research is needed to evaluate responses of various fish species subjected to exercise from water currents. These systems have been engineered to maintain water quality but little attention, if any, has been given to the rate at which the fish are forced to swim.

Salmonid species are known for migrating over vast distances and living in swiftly flowing water. Atlantic Salmon (*Salmo salar L.*) travel from fjords to the open Atlantic ocean covering hundreds of miles (Hansen *et al.* 1993, Kamalam *et al.* 2017). Rainbow trout (*Oncorhynchus mykiss*) are commonly caught by sports fisherman in flowing headwaters and streams. Swimming in a constant flow is part of the natural history of salmonids and subjection to low flow speeds of 0.5 body lengths/second (bl s^{-1}) to 1 bl s^{-1} has shown benefits in growth and immunity (Davison 1997, Grisdale-Helland *et al.* 2013). Other researchers have found that some non-salmonid marine species such as yellowtail kingfish (*Seriola lalandi*), and gilthead sea

bream (*Sparus aurata*) had improved weight gain when subjected to a current requiring them to swim (Brown *et al.* 2011, Palstra *et al.* 2015, Velez *et al.* 2016), but there has been little effort to explore the potential benefits of sustained swimming on temperate and tropical freshwater species.

Though energy expenditure is increased due to constant swimming, there are a multitude of social and physiological possibilities to explain why fish exercise may improve performance. Enhancement of muscle growth (Ibarz *et al.* 2011), reduction in the expression of social hierarchy (Laursen *et al.* 2015), an increase in carbohydrate utilization for energy (Li *et al.* 2015), improved feed efficiency, and reproduction control (Patterson *et al.* 2004, Palstra and Planas 2011) all have been suggested as potential benefits of fish exercise. However, fish are very diverse and display a wide range of activity levels, social behaviors, nutrient requirements, and reproduction strategies; therefore, the effects of exercise on fishes is likely to be species specific.

Nile tilapia is a native fish throughout Africa (Bezault *et al.* 2012) and is one of the upmost economically important protein sources for impoverished countries and tribes (Ansah *et al.* 2014) as well as one of the leading groups produced in commercial aquaculture. Worldwide, nearly 5 million tons are produced, comprising about 8% of total global fish farming (FAO 2018). Tilapia are able to withstand a wide range of conditions that are typically not suitable for other species. These include relatively high temperatures (Kaya *et al.* 2016), low dissolved oxygen levels (Abdel-Tawwab *et al.* 2014), both hard (de Holanda Cavalcante *et al.* 2014) and soft waters (Ebrahimpour *et al.* 2010) ranging from brackish to fresh in total dissolved solids (Guisheng *et al.* 2016). Because of this versatility, considerable efforts has been made to enhance tilapia aquaculture. For example, Malaysia developed genetically improved farmed tilapia

(GIFT) which have been selected for fast growth rates over 12 generations (Khaw *et al.* 2016). Unfortunately, with improved growth rates, GIFT fish are also more aggressive (Khaw *et al.* 2016). Incidences of hierarchical behavior increase energy expenditure of both aggressive and passive groups of fish, and cortisol levels in the suppressed individuals has been shown to reduce feed efficiency and growth rates (Morandini *et al.* 2014). In contrast, salmonid species raised in flowing water at slow constant speeds have shown reduced aggression towards other individuals (Sloman *et al.* 2002).

Hybrid striped bass (HSB) is a cross between the marine striped bass (*Morone saxatilis*) and the freshwater white bass (*Morone chrysops*) that is being cultured for both stock enhancement and food production. Eggs from striped bass and sperm from white bass produce the palmetto bass, and the reciprocal cross results in the sunshine bass (Ludwig 2004). Hybrid striped bass may be stocked in various habitats such as freshwater streams, lakes, and reservoirs (Axon and Whitehurst 1985) as well as cultured in ponds and recirculating tank systems with negligible or variable flow. An environment similar to that selected in the wild could prove beneficial to health and growth of HSB.

Carnivorous fish such as HSB and the red drum tend to use dietary lipids and protein more efficiently for energy than soluble carbohydrates. The reasons for limited carbohydrate utilization by carnivorous fish are multi-faceted (Kamalam *et al.* 2017). However, due to the fact that soluble carbohydrates are the least expensive source of dietary energy, it would be desirable to maximize carbohydrate inclusion in the diets of carnivorous species. There has been some evidence indicating salmonid species utilize carbohydrates more efficiently under sustained swimming (Kieffer *et al.* 1998). Therefore, potential may exist to improve utilization of dietary carbohydrates by RD and HSB in response to continuous activity as seen in salmonid species.

In-pond raceways are a culture systems which crowd high densities of fish within relatively small areas of confinement within a larger body of water (Yoo *et al.* 1995). A constant circulation of water, discharged by a set of airlifts, delivers oxygen rich water and removes nitrogenous waste from the enclosure while simultaneously creating a slow current (Masser *et al.* 1993). Feed efficiency and production of fish cultured in such in-pond raceways may be drastically improved for various suggested reasons (Brown *et al.* 2011, Yoo *et al.* 1995), but research has yet to focus on the speed of the current against which the fish are forced to swim. Improved production from in-pond raceways may entice more commercial facilities to implement such systems, and having a better understanding of the effects of exercise due to water current in these systems is needed.

II.2 Materials and Methods

System Construction

Four round fiberglass tanks of 1.22-m diameter (inner tank) were fitted inside four 1.52-m diameter (outer tank) round fiberglass tanks to produce chambers operating under controlled conditions for exercising fish with regulated water flow. Inside the inner tank, a 0.46-m cylinder housed a ¼ horse power pump (FLOTEC 4/10 HP SUBMERSIBLE MODEL FP0S3000X). This setup allowed for two swimming areas, 0.3-m (exterior area) between the two tanks and 0.76-m (interior area) between the inner tank and cylinder. Before fixing the inner tank, the outer edge of the bottom was lined with weather stripping. The bottoms of both tanks, as with most round tanks, had a slight conical slope and the weather stripping prevented fish from escaping current and swimming under the inner tank. A 5.1-cm and 2.5-cm diameter hole at the top of the inner tank and cylinder, respectively, prevented overflow. A 10.2-cm hole was drilled in the center of

the inner tank giving space for a stand pipe. A mesh lid on top of the cylinder prevented fish from jumping into the cylinder.

Inside the cylinder, a submersible pump was plumbed with polyvinylchloride (PVC) pipe extending above the cylinder. A four-way fitting was attached and four PVC lines were extended to the edge, then bottom, of the inner and outer tanks. The number of nozzles reaching the inner and outer tanks were used to assist in determining the rate of flow in the swimming area and also create consistent flow throughout. To further adjust flow, the plumbing to each nozzle incorporated a valve.

Fish

Three different warm-water fish cultured in Texas and elsewhere were evaluated in three separate trials in this study. Hybrid striped bass were obtained from Keo Fish Farms (Lonoke, Arkansas) and transported via truck to the Texas A&M University Aquacultural Research and Teaching Facility. Red drum were transported from Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center operated by Texas Parks and Wildlife Department in Lake Jackson, TX. Nile tilapia were bred and raised at the Texas A&M Aquacultural Research and Teaching Facility.

Exercise Trials

A week prior to the beginning of each trial, tilapia, RD, and HSB, approximately 19 cm in length, were implanted with passively integrated transponder (PIT) tags and acclimated to a recirculating system held at 5 ppt total dissolved solids. Fish were then individually weighed, measured, and PIT-tag number recorded before randomly distributing to tanks operated

according to either exercise or static regimens. Tilapia are dimorphic considerably early in life, therefore, they were visually sexed to assure all fish are male; whereas, RD and HSB were not sorted by sex.

Exercise Regimens

In separate feeding trials, 20 HSB, 22 tilapia, or 34 RD, were equally divided and distributed into flowing or static tanks. Fish in the flowing tanks were subjected to a flow rate requiring a swimming speed of 1.0 bl/s. Speed of current was calibrated via flowmeter (Flo-Matetm Marsh McBirney Model 2000 Portable Flowmeter) based on the average speed taken from nine separate positions of the inner swimming area and six separate positions of the outer swimming area. The water speed was kept constant during the trial and thus swimming speeds, relative to fish length, were slightly decreased over time (Grisdale-Helland *et al.* 2013). Final length of individual fish was measured and relative speeds documented at the end of the trial. Exercise or static conditions were imposed continuously (24 h per day) and each fish represented a replicate observation.

Throughout each trial, fish were fed to apparent satiation twice daily. This was achieved by providing feed to each tank in the morning and evening and carefully monitoring intake. Feed was provided to each tank over a 15-minute period until feeding activity was reduced and no excess feed remained in each tank.

At the end of each 7-week feeding trial, all fish were collected from each tank, weighed and then anesthetized with tricaine methane sulphonate (MS-222, 150 mg L^{-1}). Three fish per tank where processed for body condition indices including hepatosomatic index (HSI), intraperitoneal fat (IPF) ratio (IPF ratio) and muscle yield (MY). Four fish per tank were

processed for whole-body proximate composition. Dry matter was determined by placing ingredient samples in an oven at 135 °C for 2 h (AOAC 1991). Ash values will be determined by subjecting the samples to 600 °C for 3 h (AOAC 1990) (AOAC 1991). Crude protein was be estimated by measuring total nitrogen by the Dumas method (Ebeling 1968) and multiplying by 6.25. Lipid was determined by the chloroform/methanol extraction method described by (Folch *et al.* 1957). Gross energy was analyzed by an adiabatic micro-bomb calorimeter (AOAC 1990) (AOAC 1991).

Calculations and statistical analyses

The following fish performance variables were utilized to compare treatments:

Weight gain (%) = [(final body weight [BW], g) − (initial BW, g)/ (initial BW, g)] ×100;

Feed efficiency ratio (FE) = [weight gain (g)/ dry feed consumed (g)] Muscle yield (%) = [(fillet muscle weight, g)/ (BW, g)] ×100;

Viscerosomatic indices (HSI or IPF ratio, %) = [(liver or IPF weight, g)/ (BW, g)] ×100.

All evaluated variables for RD, HSB and tilapia were separately subjected to an analysis of variance (ANOVA) to determine if exercise affected the observed responses. Statistical analyses were conducted using JMP of the Statistical Analysis System (version 9.0, SAS Institute, Cary, NC, USA). Differences amongst treatment means were considered significant at $P<0.05$.

II.3 Results

Male tilapia outgrow female tilapia and are the preferred sex to culture to a marketable size (200-600 grams). Therefore, in the current study, all male tilapia were visually sexed via

their urogenital opening. Both exercised (average = 291 g) and static tilapia (average = 231 g) grew to minimum market size over the duration of the trial.

Results from this study indicate that continuous exercise negatively affected growth of HSB, possibly due to consistent skittish feeding behavior. In contrast, significant ($P \leq 0.05$) improvements were observed in % weight gain of exercised red drum and tilapia compared to control treatments (Table 1). Survival was not affected by exercise in any species and was 100% for hybrid striped bass and tilapia.

There were no significant differences observed for intraperitoneal fat (IPF) ratio of exercised versus static fish of any species evaluated in this study (Table 2). However, some marked differences in the amount of IPF deposited by the various fish were noted with HSB depositing much more IPF than red drum or tilapia (Table 2). Some differences in proximate composition of whole-body tissues were observed for the various fish species. Exercised fish of all species showed increased whole-body protein percentage although for RD the increase was not quite statistically significant at $P = 0.07$ (Table 2). A significant decrease in whole-body moisture was observed in exercised RD and tilapia but not HSB (Table 2). All species showed differences in whole-body lipid percentage due to exercise, with static HSB having greater whole-body lipid; whereas, exercised RD and tilapia had lower whole-body lipid (Table 2). There were no differences in whole-body ash percentage except for tilapia in which ash content increased in fish maintained in the static system.

Hepatosomatic index and liver glycogen, lipid and protein were responsive to exercise but varied depending on fish species (Table 3). Hepatosomatic index values were similar for the various species and lower in all exercised fish compared to static fish; however, differences were not significant for RD or tilapia (Table 3). In contrast to HSI, significant differences were

detected in liver glycogen of exercised and static tilapia and RD, with glycogen increasing in exercised tilapia but decreasing in red drum. Liver glycogen also tended to be reduced in exercised HSB although differences were not statistically significant (Table 3). All species exhibited a significantly higher percentage of liver lipid in exercised compared to static fish (Table 3). Liver protein was significantly increased only in exercised red drum compared to the other fish species (Table 3).

Muscle yield and muscle proximate composition were generally not as responsive to exercise compared to whole-body or liver tissues (Table 4). Muscle yield of static HSB was significantly greater than exercised HSB, but there were no differences detected in RD or tilapia. There were no significant differences in the lipid, protein, ash, or glycogen percentages in the muscle of any fish species due to exercise although there was a trending ($P<0.06$) decrease in muscle lipid and increase in white muscle glycogen of exercised RD (Table 4). It also should be noted that there was a trend ($P<0.08$-0.10) for increased red muscle glycogen in exercised fish of all species.

II.4 Discussion

As seen in this study, while there was overlap among species, responses to exercise were species specific and confounding conditions such as photoperiod, temperature, and behavior may have influenced responses of the different fish. Poor growth performance of HSB in this experiment is a prime example. In a previous study, (Young and Cech Jr 1994) observed improved performance of active HSB forced to swim at speeds from 0.5 bl/s to 3.6 bl/s. However, in this study, HSB swimming at 1 bl/s had a significant reduction in weight gain. There are several differences between the two studies including system construction, starting

Table 1. Growth performance of exercised and static hybrid striped bass (HSB), red drum and tilapia.

	Initial Weight Grams	% Weight Gain	Feed Efficiency	Survival
HSB				
Exercise	77.3	125[b]	0.66	100
Static	78.7	155[a]	0.81	100
P>F	0.4522	0.0023	-	-
Pooled Std Error	1.2847	5.6826	-	-
Red Drum				
Exercise	74.6	156[a]	0.69	88
Static	75.1	120[b]	0.58	82
P>F	0.6591	.0002	-	-
Pooled Std Error	0.79301	6.2818	-	-
Tilapia				
Exercise	96.7	202[a]	0.79	100
Static	99.1	133[b]	0.71	100
P>F	0.2290	.0007	-	-
Pooled Std Error	1.3628	10.068	-	-

Table 2. Whole-body composition of exercised and static hybrid striped bass (HSB), red drum and tilapia.

	IPF	% Moisture	Protein	Lipid	Ash
HSB					
Exercise	6.08	66.8	17.62[a]	11.55[b]	9.45
Static	6.38	66.6	15.96[b]	13.98[a]	9.98
P>F	0.5395	0.6191	0.0019	0.0019	0.0785
Pooled Std Error	0.33173	0.22944	0.22215	0.32675	0.17694
Red Drum					
Exercise	0.95	72.4[b]	17.69	7.47[a]	12.45
Static	1.29	74[a]	17.19	6.02[b]	12.28
P>F	0.3149	0.0009	0.0713	0.0001	0.2449
Pooled Std Error	0.23014	0.19543	0.16158	0.11815	0.09327
Tilapia					
Exercise	2.03	69.6[b]	18.93[a]	10.26[a]	12.26[b]
Static	1.51	72.4[a]	15.87[b]	8.08[b]	14.52[a]
P>F	0.3054	0.0002	0.0001	0.0033	0.0001
Pooled Std Error	0.3369	0.25063	0.12974	0.32736	0.12045

Table 3. Hepatosomatic index and liver composition of exercised and static hybrid striped bass HSB, red drum and tilapia.

	HSI	% Liver Glycogen	% Liver Lipid	% Liver Protein
HSB				
Exercise	2.13[b]	9.3	24.23[a]	26.46
Static	2.51[a]	12.5	20.53[b]	25.09
P>F	0.0082	0.1215	0.0018	0.1460
Pooled Std Error	0.08234	1.2563	0.49427	0.58017
Red Drum				
Exercise	2.29	2.5[b]	65.3[a]	20.03[a]
Static	2.84	6.4[a]	58.25[b]	15.58[b]
P>F	0.1479	0.0001	0.0077	0.0169
Pooled Std Error	0.2478	0.16330	1.2677	0.95990
Tilapia				
Exercise	2.03	4.5[a]	28.12[a]	36.96
Static	2.21	3.0[b]	26.3[b]	35.18
P>F	0.45	0.0007	0.0099	0.1564
Pooled Std Error	0.16359	0.16731	0.34654	0.77698

initial length, and feeding methods. The reduction in growth of HSB in the current study is likely due to system construction invoking skittish feeding behavior not shared by the tilapia or RD.

Feeding Behavior

The three different fish species in the current study exhibited different feeding behaviors. On approach, RD and tilapia moved toward the water surface while HSB attempted to hide behind the 0.46-m cylinder. Once feeding started, RD and tilapia aggressively fed as diet hit the water while HSB lunged as pellets fell through the water column then quickly retreated behind the 0.46-m cylinder. Feeding behavior of HSB did not change throughout the feeding sessions. Once pellets fell to the bottom of the tank, tilapia became disinterested in pellets added near the surface, but instead, swam in random directions (against and with the current) consuming feed from the bottom. The RD aggregated against the current and began to lung for pellets similar to HSB, but without immediately retreating behind the 0.46-m cylinder. Skittish behavior demonstrated by HSB during exercise is a likely reason for poor performance.

Weight Gain

Exercise in several fish species has been shown to result in improvements in weight gain as well as other beneficial responses. For example, (Brown *et al.* 2011) found a 10% increase in weight gain (Davie *et al.* 1986), and (Davison 1997) discussed improvements in protein synthesis, FCR, and an increase in growth hormone in response to relatively low (1 – 1.5 bl/s) sustained speeds. Atlantic salmon were reported to experience similar improvements (Davie *et al.* 1986) including enhanced disease resistance when subjected to 1 bl/s (Grisdale-Helland *et al.* 2013). Most research has been focused on salmonid species (Castro *et al.* 2011, Grisdale-Helland

Table 4. Muscle yield and composition of exercised and static hybrid striped bass (HSB), red drum and tilapia.

	Muscle Yield	Protein	Lipid	Red Muscle Glycogen	White Muscle Glycogen
HSB					
Exercise	30.43[b]	20.56	14.54	0.38	0.12
Static	32.88[a]	20.42	12.23	0.30	0.22
P>F	0.0015	0.3964	0.1204	0.0964	0.0616
Pooled Std Error	0.20045	0.10432	0.82996	0.02872	0.03082
Red Drum					
Exercise	28.73	20.07	6.04	0.35	0.21
Static	29.84	20.97	7.98	0.29	0.14
P>F	0.2517	0.2641	0.0601	0.0924	0.0653
Pooled Std Error	0.32128	0.49029	0.52786	0.02121	0.02198
Tilapia					
Exercise	28.06	19.34	10.37	0.36	0.18
Static	26.46	19.53	9.19	0.24	0.15
P>F	0.2252	0.4885	0.1046	0.0823	0.0933
Pooled Std Error	0.35432	0.05568	0.39879	0.04072	0.01064

et al. 2013, Kiessling *et al.* 1994, Laursen *et al.* 2015, Parker and Barnes 2014, Patterson *et al.* 2004, Sloman *et al.* 2002) due to their aquaculture importance and natural history. Young and Cech Jr (1994) exercised HSB in a chamber which was cube shaped, fish where young-of-the-year fingerlings, and flow was stopped before feeding. In the present study, juvenile fishes where exercised in a circular chamber, and diet was fed with flow. Although HSB and RD have distinctly different natural history and environmental adaptations compared to salmonids, they are more similar to salmonids than tilapia. Nevertheless, tilapia showed the largest improvement in weight gain in the current study. Tilapia are a wide-body species and a 19-cm long tilapia is sexually mature and considerably larger (97.9 g) than RD (74.9 g) and HSB (78.0 g) at the same length. Fishes with greater body weight to length ratio have been reported to have higher optimal swimming speeds on a bl/s basis (Palstra *et al.* 2015, Vagner *et al.* 2008, Palstra and van den Thillart 2010, Tudorache *et al.* 2011). Typically, intraspecific comparisons of exercise vs static fish are evaluated based on an initial standardized parameter of total length or fork length. Interspecific comparisons should compare animals of similar environmental or economic importance and other initial standard parameters such as weight or physiological development should be considered.

Tilapia are a deep-body fish and lack the streamline characteristics of juvenile HSB and RD. Despite their girth, tilapia adapted well to exercise, and their performance under exercise showed that morphology should not exclude species from consideration. Similarly, Cleujosí da Silva *et al.* (2013) exercised pacu *(Piaractus mesopotamicus)*, a deep bodied species, at 2 bl/s and saw an increase in weight gain. In another study by Li *et al.* (2015), tilapia exercised by swimming at 1.5 bl/s increased the activity of glycolytic enzymes and reduced lipid oxidation for

energy metabolism, indicating an increase in the use of carbohydrates. The present study found exercised tilapia tended to have higher glycogen percentages in red and white muscle tissues.

Body condition and composition

Body composition has been the focus of research for many fishes (Dumas *et al.* 2010) (Bender *et al.* 2014) (Breck 2014). Distribution of nutrient constituents can influence growth, energy metabolism, health, and adaptability. For example, polyunsaturated fats added to the diets of fishes alter the body fatty acid composition thus improving cold tolerance of hybrid striped bass (Kelly and Kohler 1999), red drum (Craig *et al.* 1994) and tilapia (Correa *et al.* 2017). In the current study, exercise altered lipid deposition of each species by increasing the accumulation of lipids in the liver. Red drum had dramatically greater liver lipid percentage than HSB and tilapia and can develop fatty liver syndrome (Craig *et al.* 1999). Differences in lipid distribution for exercised red drum is possibly due to the mobilization of carbohydrates from the liver to both red and white muscle tissue, but the potential of exercise redistributing lipid deposition could play a role in some aspects of lipid metabolism and a potential avenue of future research with RD. For all species, exercised fish had smaller, but fattier livers, likely due to the mobilization of glycogen from the liver.

There was a reduction in HSI amongst exercised groups of all fish species in this study although only HSB proved to be significant. Several studies have reported numerical HSI reduction in salmonid species as an effect of sustained exercise (Parker and Barnes 2015, Kiessling *et al.* 1994, Parker and Barnes 2014), likely due to increased glycogen utilization. Studies with Atlantic cod (*Gadus, morhua L.*) (Bjornevik *et al.* 2003, Karlsen *et al.* 2006) saw an initial reduction in HSI in response to 0.5-1 bl/s exercise but, as the fish grew, exercised fish HSI

caught up, indicating that fish adapted over time or larger fish more easily swim in respectively slower current. In the present study, the tendency for lower levels of glycogen in the liver of exercised RD and HSB likely relates to the reduction in their HSI, but this was not the case for tilapia where static fish had significantly lower liver glycogen percentage.

Exercise is widely known to increase muscle glycogen storage and glycogen metabolism (Hargreaves 1997, Hansen *et al.* 2005). This generally holds true in fish (Jobling *et al.* 1993) and was generally reaffirmed in the current study. Post-prandial hyperglycemia in response to high dietary levels of soluble carbohydrates is common in fishes (Kamalam *et al.* 2017). In a study of rainbow trout, Felip *et al.* (2011) found that exercise improved the deposition of glycogen in muscle tissue due to the up-regulation of genes involved in glycogen metabolism. Therefore, exercise improved the storage and transportation of glycogen inside muscle tissue improving carbohydrate utilization. The tendency for increased red muscle glycogen stores was observed for all species of this study, generally indicating an increase in carbohydrate utilization (West *et al.* 1993). A tendency for increased white muscle glycogen also was found in RD and tilapia but not HSB in the present study.

CHAPTER III

EFFECTS OF DIETARY CREATINE ON CHANNEL CATFISH FRY IN WATERS OF VARIOUS SALINITIES

III.1 Introduction

Quantified amino acid requirements are important for precisely formulating diets of fishes and as such have been heavily researched for species with a long history of aquacultural production (Nunes *et al.* 2014). Much less research has evaluated the importance of various organic acids and other products of amino acid metabolism.

Creatine is an organic acid synthesized in the body from arginine, methionine, and glycine. Although creatine can be synthesized by the body, supplementation of creatine in the diet of humans (Volek and Kraemer 1996), rats (Antolic *et al.* 2007), chickens (Stahl *et al.* 2003) and pigs (Maddock *et al.* 2002) have shown to improve muscular growth. However, due to an inefficient ability to absorb creatine (Sewell and Harris 1995), evidence suggests that horses do not benefit from oral creatine supplementation (Geor 2007). Research on creatine metabolism of fish has been very limited to date (Danulat and Hochachka 1989, Weng *et al.* 2002).

Biosynthesis of creatine in the liver and kidney of mammals is well understood (Joncquel-Chevalier Curt *et al.* 2015). In fish, Wang *et al.* (2010) found creatine biosynthesis in zebrafish (*Danio rerio*) to be similar to that of mammals. Yet, primary enzymes for creatine biosynthesis in rainbow trout (*Oncorhynchus mykiss*) have been found in intermuscular tissue (Borchel *et al.* 2014). Uncertainty of creatine biosynthesis in fish (Mommsen 2001) but the general benefit of dietary creatine in many animals puts in question the potential benefit of

dietary creatine supplementation for fish, especially under conditions which are energy demanding such as under osmoregulatory stress.

The majority of creatine is stored in muscle tissue and primarily functions as a regenerative energy source and storage form of phosphate (Borchel *et al.* 2014). Creatine is also found in the gills of fish where it plays a similar role in the energetics of osmoregulation (Ching-Feng *et al.* 2002). Fishes maintain constant plasma osmolarity by actively pumping Na and K ions to and from their environment through their gills (Zohouri *et al.* 2001) or ingesting ions from their diet (Dabrowski *et al.* 1986, Gatlin *et al.* 1992). Supplemental creatine has the potential to have ergogenic benefits due to the increase in creatine kinase activity during times of osmotic stress (Kultz and Somero 1995, Ching-Feng *et al.* 2002, Lin *et al.* 2003). Stenohaline fish such as channel catfish, when raised in salinities not consistent to their natural environment, go through osmotic stress that can cause a reduction in growth performance (Allen and Avault Jr 1971) or even cause death (Allen 1971). Some dietary supplements have been shown to alleviate osmotic stress and improve osmoregulatory ability in fishes. For example, dietary arachidonic acid was found to increase expression of NA/K ATPase, an active osmoregulatory enzyme (Carrier *et al.* 2011). Several studies have demonstrated the growth benefits of adding salt to the diets of marine fish raised in brackish water (Salman and Eddy 1988, Gatlin *et al.* 1992, Pyle *et al.* 2003) . Supplemental ions such as calcium, phosphorus, and magnesium also have demonstrated osmoregulatory benefits in fishes (Baldisserotto *et al.* 2007).

Channel catfish are a freshwater fish primarily cultured in the southern region of the United States and is considered a stenohaline species that grows best in waters of 0 to 5 ppt (Allen 1971). Allen (1971) found that survival and growth performance of channel catfish was dramatically decreased at 9 ppt, and mortality of 40-day-old fry was 100% when cultured at 12

ppt for 17 days. Yet, raising channel catfish in low-salinity water can affect their susceptibility to freshwater parasites and pathogens such as *Ichthyophthirius multifilis* (Allen and Avault 1970), *Flavobacterium columnaris* (Altinok and Grizzle 2001), and *Edwardsiella ictaluri* (Plumb and Shoemaker 1995). These pathogens may be effectively treated by an increase in salinity; therefore, formulating a diet to assist with osmotic stress (Gatlin *et al.* 1992) during salt treatment of pathogens may improve outcomes for species such as channel catfish. Based on these various reasons, this study was conducted to examine the potential benefit of supplementary creatine on the osmoregulation of channel catfish cultured under different water salinity conditions.

III.2 Materials and Methods

Two feeding trials were conducted to examine the effects of supplementary creatine on weight gain, feed efficiency and survival of channel catfish fry in waters of varying saline. For both feeding experiments, channel catfish fry were produced at the Texas A&M Aquacultural Research and Teaching Facility. Eggs were retrieved from spawning receptacles in ponds, then treated with sodium sulfite to remove gelatinous matrix before being placed in McDonald jars until they hatched. Each trial was conducted with a single separate cohort of catfish fry.

Trial I

The basal diet for this trial was formulated to contain 40% crude protein, contributed equally by menhaden fishmeal and soy protein concentrate, and 10% lipid, primarily from menhaden fish oil (Table 5). The experimental diet was derived from the basal diet by

supplementing creatine at 2% by weight at the expense of cellulose, and adjusting a 50/50 blend of aspartate and glutamate to maintain the diets isonitrogenous.

Channel catfish fry were acclimated for 1 week in six, 38-L aquaria operated as a recirculating system and fed the basal diet before the start of the trial. Each aquarium was stocked with 25 fish with an initial average weight of 0.29 ± 0.08 g/fish. The basal diet and experimental diet were each randomly assigned to three aquaria. Salinity of the recirculating water system was maintained at 7-8 ppt by adding synthetic sea water. Duration of trial I was 8 weeks.

Trial II

In the second feeding trial, channel catfish fry were conditioned on a commercial diet containing 54% crude protein and 17.1% lipid on a dry-matter basis (Rangen, Inc.) and were acclimated for 1 week in eight, 110-L aquaria connected as a recirculating system. The experimental diet was produced by adding creatine (to provide 2 g/kg on a dry-matter basis) in gelatin powder dissolved in water and coating the mixture onto the commercial diet.

Each aquarium was stocked with 50 channel catfish fry with an initial average weight of 0.4 g/fish. The commercial diet and a 2% creatine coated diet was each randomly assigned to four aquaria.

The water salinity for the first 6 weeks of trial II was maintained at 2 ppt with a mixture of sodium chloride and artificial sea water. After 6 weeks, the fish were weighed, and the salinity was increased to 10 ppt with the same artificial seawater mixture and the trial continued for an additional 4 weeks after which the fish were sampled.

In both feeding trials, water temperature was maintained at 26 ± 1 C and dissolved

Table 5. Formulation and proximate composition of experimental diets fed in trial I.

	Diet	
Ingredient	basal	2% creatine
Menhaden meal	29.0	29.0
Soy protein conc.	28.0	28.0
Dex. starch	22.0	22.0
Menhaden oil	6.3	6.3
Vitamin premix	3.0	3.0
Mineral premix	4.0	4.0
Carboxymethyl cellulose	2.0	2.0
Creatine	0.0	2.0
Cellulose	5.7	3.7
Analyzed proximate composition (% dry weight)[a]		
Dry matter	89.2	89.1
Crude protein	40.8	41.4
Crude lipid	10.2	10.3
Ash	6.6	6.5
Creatine	0.1	2.1

[a] Values are means of duplicate analyses.

oxygen kept at air saturation with air from a blower passed through air stones in each aquarium. A 12h light: 12h dark photoperiod was maintained using fluorescent lighting controlled by timers. Also, in both trials the fish were fed at 6% of body weight daily, divided into two feedings. Each tank of fish was collectively weighed weekly and feed rations were adjusted accordingly.

At the end of each feeding trial, six fish from each aquarium were anesthetized with tricaine methane sulfonate (MS-222, 150 mg L^{-1}) and frozen for whole-body proximate composition. Six additional fish were anesthetized as previously described, weighed and dissected to obtain liver, and intraperitoneal fat (IPF) for computing hepatosomatic index (HSI) and IPF ratio, respectively. Muscle tissue samples also were obtained for proximate composition and creatine analysis. Creatine was analyzed in diets and muscle tissues using the Jaffe reaction as described by (Bonsnes and Taussky 1945).

Calculations and statistical analyses

The responses utilized to compare treatments in this study were calculated as follow: Weight gain, % = [(Final weight – initial weight)/(initial weight)] × 100;
Feed efficiency ratio (FE) = [weight gain (g)/ dry feed consumed (g)] Survival = [initial # of fish/ final # of fish] x 100; Viscerosomatic indices (HSI or IPF ratio, %) = [(liver or IPF weight, g)/ (BW, g)] ×100. The responses of fish in each feeding trial was analyzed using one-way analysis of variance using the Statistical Analysis System (version 9.0, SAS Institute, Cary, NC, USA). Significance was set at 5%.

III.3 Results

Trial I

Channel catfish fry fed the diet supplemented with 2% creatine in brackish water resulted in significant differences in weight gain and feed efficiency but not in HSI, IPF, or survival (Table 6). Whole-body proximate composition revealed a trending differences ($P<0.1$) in moisture, but no significant differences in crude protein, crude lipid or ash (Table 7). There also was no significant difference in muscle crude protein, crude lipid or ash (Table 7). However, a significant difference was found in muscle creatine, indicating channel catfish were able to take up dietary creatine and deposit in their muscle.

Trial II

There were no significant differences in weight gain, feed efficiency, HSI, or IPF of channel catfish fry fed the two diets during the freshwater, brackish water or the cumulative phases of feeding trial II (Table 8). The survival of catfish fry in the freshwater phase was not significantly different; however, after raising the salinity, there was a slight but significant reduction in survival of fry fed the basal diet (Table 8). Similar to Trial I, whole-body and muscle proximate composition showed no significant differences in moisture, crude protein, crude lipid, or ash (Table 9). However, there was a significant difference in muscle creatine (Table 9).

Table 6. Growth performance and body condition indices of channel catfish fry (initially averaging 0.29 g/fish) fed diets with and without supplemented creatine for 8 weeks in Trial I.[a]

	Diet			
	Basal	2% Creatine	P-Value	Pooled Std Error
Initial Biomass, g/aquarium	7.2	7.1	0.7376	0.26247
Weight gain, % of initial weight	2169[b]	2595[a]	0.0249	86.069
Feed efficiency	0.655[b]	0.72[a]	0.0285	0.1385
Hepatosomatic index	1.41	1.43	0.5571	0.02489
Intraperitoneal fat ratio	3.08	3.31	0.3839	0.18167
Survival, %	99	100	0.3739	0.94281

[a]Values are means of three replicate groups.

Table 7. Proximate composition (% of fresh weight) of channel catfish fry fed diets with and without creatine supplementation for 8 weeks in Trial I.[a]

	Diet			
	Basal Diet	2% Creatine diet	P-Value	Pooled Std Error
Whole-body				
Moisture	69.3	69.7	0.083	0.13222
Crude protein	17	17.3	0.1673	0.1119
Crude lipid	7.7	7.5	0.5012	0.19149
Ash	3.9	3.8	0.5342	0.09715
Muscle				
Moisture	80.9	80.4	0.2455	0.24267
Crude protein	17.5	17.9	0.2239	0.17544
Crude lipid	3.7	3.6	0.587	0.15986
Creatine	0.341[b]	0.407[a]	0.0045	0.10612

[a]Values are means of three replicate groups.

Table 8. Growth performance of channel catfish fry (initial average weight of 0.4 g/fish) fed a commercial diet with or without creatine supplementation for 6 weeks in freshwater (FW) and 4 weeks in brackish water (BW) in Trial II.[a]

	Diet			
	Commercial Diet	2% Creatine diet	P-Value	Pooled Std Error
Initial biomass/aquarium (g)	20.3	20	0.9046	1.8379
Initial biomass/aquarium (BW)	96	100	0.6243	5.8245
% Weight gain (FW)	411	430	0.8097	52.895
% Weight gain (BW)	114	119	0.4319	4.0207
% Total weight Gain	996	1062	0.7136	119.91
Feed efficiency (FW)	0.59	0.63	0.4228	0.03207
Feed efficiency (BW)	0.58	0.64	0.1443	0.02739
Total feed efficiency	0.583	0.638	0.1936	0.02645
Hepatosomatic index	1.39	1.46	0.214	0.05
Intraperitoneal fat ratio	2.68	3.01	0.183	0.4136
% Survival (FW)	96	96	1	2.2174
% Survival (BW)	94[b]	99[a]	0.0428	1.2416
% Survival (10 wk.)	90	94	0.4055	3.1623

[a]Values are means of four replicate groups.

Table 9. Proximate composition (% of fresh weight) of channel catfish fry fed a commercial diet with and without creatine supplementation for 10 weeks in Trial II.[a]

	Diet			
	Commercial Diet	2% Creatine diet	P-Value	Pooled Std Error
Whole-body				
Moisture	69.8	69.6	0.328	0.8641
Crude protein	17.2	17.1	0.923	0.4312
Crude lipid	7.3	7.6	0.411	0.6125
Ash	3.2	3.6	0.085	0.2231
Muscle				
Moisture	80.7	80.6	0.917	0.29787
Crude protein	17.8	18.4	0.131	0.3521
Crude lipid	3.6	3.5	0.256	0.2428
Creatine	0.365[b]	0.425[a]	0.0202	0.01354

[a]Values are means of four replicate groups.

III.4 Discussion

Channel catfish fed creatine displayed significantly improved weight gain and feed efficiency in trial I but not in either the FW or BW period of trial II. However, growth rate of fish in trial II dramatically decreased once water salinity was increased to 10 ppt. Although fish in trial II had a larger initial starting weight (0.4 g/fish) and the trial lasted for 10 weeks, the smaller initial size (0.29 g/fish) of fish in trial I had a greater individual final weight (7.1 g/fish) after 7 weeks compared to those from trial II (4.4 g/fish). Allen (1971) found that survival and growth performance of channel catfish was dramatically decreased at 9 ppt, and mortality of 40-day-old fry was 100% when cultured at 12 ppt for 17 days. This study reaffirms those results as catfish fry in trial I and those during the FW period in trial II had survival that was 99% and 96%, respectively. After the salinity increased in trial II, catfish fed the diet supplemented with creatine had better survival compared to those fed the commercial diet without creatine supplementation. These results suggest creatine supplementation is beneficial to relieve osmotic stress of channel catfish.

Osmotic stress can be detrimental to growth and survival of channel catfish, and though creatine does not contribute as a substrate for energy, Creatine - creatine phosphate shuttle, where creatine acts as a regenerative source of phosphate for ATP, has been recognized in the gills of fish (Kultz and Somero 1995),(Lin *et al.* 2003). Thus, gills could potentially take up circulating creatine, and energetically assist in osmoregulation.

Significant increases in muscle creatine of channel catfish fed supplemental creatine indicate a mechanism for transport and deposition of exogenous creatine to muscle tissue. Results of this study also suggest supplemental creatine improves the robustness of channel

catfish during osmotic stress. As such, the supplementation of creatine to the diet may be viewed as conditionally required by channel catfish in brackish water.

Conclusions

Creatine supplementation improved the performance of channel catfish raised in brackish waters of 7 ppt or higher. Muscle creatine percentage indicated dietary creatine was readily deposited in the muscle of channel catfish fish. Although creatine may be cost prohibitive to include in the regular diet of channel catfish reared in FW, diets consisting of 2% creatine may benefit fish in times of BW influx or during BW disease treatment.

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CHAPTER IV

EFFECTS OF DIETARY CREATINE ON JUVENILE HYBRID STRIPED BASS IN LOW-SALINITY AND BRACKISH WATERS

IV.1 Introduction

Hybrid striped bass (HSB) is a cross between white bass and striped bass resulting in a euryhaline carnivorous fish which is cultured in the U.S., Asia, and Europe for recreational fishing and seafood production (Wattendorf and Shafland 1982, Harrell and Webster 1997, Hiney *et al.* 2002). Likely due to the crossing of a freshwater and saltwater species, and the anadromous nature of striped bass, effects of salinity on HSB has been a point of focus for several researchers (Wattendorf and Shafland 1982, Woods III *et al.* 1983, Hodson 1990, Brown *et al.* 1992, Bielmyer *et al.* 2005). Tomasso (1997) found that HSB require different salinities during development, and salinity tolerance of moronids increases with development (Stickney 1992). Survival of fry is dramatically decreased at salinities higher than 9 g/L, but juvenile HSB survival was not affected at salinities below 20 g/L (Myers and Kohler 2000). Weight gain of juvenile hybrid striped bass was severely reduced when cultured at 32 g/L compared to 7 or 0 g/L which consequently affected their dietary protein requirement (Brown *et al.* 1992). Salinity effects on performance of fishes interact with several environmental factors including temperature (Buckel *et al.* 1995, Imsland *et al.* 2001, Fiess *et al.* 2007) and hardness (Mazik *et al.* 1991, Michelotti *et al.* 2018), and diet (Alava 1998, Salze and Davis 2015). Though research has focused on some of these components of salinity tolerance, other information on osmoregulation and energy requirements of the cultured fish is required to understand how these factors interact (Boeuf and Payan 2001).

Fishes actively uptake ions from hypotonic environments and discharge ions from hypertonic environment to maintain homeostasis. In osmotically challenging mediums, fishes experience osmotic stress and alter the number of chloride cells in the gill as well as enzymatically adjust (McCormick 2001, Hirose *et al.* 2003). To measure osmotic stress, studies have measured serum osmolality (Skadhauge 1969, Laverty and Skadhauge 2012) and Na/K-ATPase (NKA) activity (Epstein *et al.* 1967, McCormick 1996, Weng *et al.* 2002) in gills and intestine of fish, and have shown that acclimation to salinity change is energy consuming and increases metabolic rate (Laverty and Skadhauge 2012). Several marine species have benefited from dietary salt supplementation while raised in hyposaline environments, indicating there is potential for nutrition to alleviate osmotic stress (Harris Jr *et al.* 2002, Harpaz *et al.* 2005).

A creatine-creatine phosphate shuttle has been recognized in fish gills (Kultz and Somero 1995, Lin *et al.* 2003), and much like NKA, creatine kinase activity increases during times of osmotic stress (Weng *et al.* 2002). Therefore, there is potential for dietary creatine to support ion exchange for osmoregulation.

Creatine supplementation has been shown to affect growth, immunity, and ergogenic responses in mammals (Volek and Rawson 2004, O'Quinn *et al.* 2000). In fish, dietary creatine supplementation has been shown to affect sprint endurance (McFarlane *et al.* 2001) and osmoregulation (Weng *et al.* 2002). However, very limited research has addressed the effects of creatine on growth of fish. This is likely because creatine is considered to be a non-essential organic acid that is not typically supplemented in diet formulations. Unlike non-essential amino acids, creatine degrades into creatinine and is excreted, and therefore is not catabolized to produce ATP (Mesa *et al.* 2002). Yet, creatine kinase catabolizes creatine phosphate to produce ATP during times of high energy demand (Wyss and Kaddurah-Daouk 2000). Therefore, dietary

supplementation of creatine may confer benefits to fish experiencing osmoregulatory stress. This hypothesis was tested with HSB cultured in brackish water where additional energy is typically required for osmoregulation compared to water of lower salinity. Therefore, this study evaluated dietary creatine supplementation on HSB cultured in fresh and brackish waters to evaluate any conditional requirement for dietary creatine based on osmotic conditions.

IV.2 Materials and Methods

A two-part feeding trial was conducted to examine the effect of supplementary creatine in the diet on growth performance of hybrid striped bass (HSB) in low-salinity (3 g/L) and brackish water (15 g/L). Juvenile HSB were obtained from Keo Fish Farms (Lonoke, Arkansas) and transported via truck to the Texas A&M University Aquacultural Research and Teaching Facility. Fish were cultured in a recirculating system with water temperature maintained at 26 C and dissolved oxygen was close to air saturation. A 12 h light:12 h dark photoperiod was maintained using fluorescent lighting controlled by timers.

Fish were conditioned on a commercial diet (Rangen, Inc.), containing 40% crude protein and 12% lipid, and were acclimated for 2 weeks in 20, 110-liter aquaria connected as a single recirculating system. At the beginning of the feeding trial, each aquarium had 18 fish with an individual initial average weight of 2.2 g/fish.

A basal diet was formulated to contain 40% crude protein, contributed equally by menhaden fishmeal and soy protein concentrate, and 10% lipid. Three experimental diets were derived from the basal diet by supplementing creatine at 1, 2, or 4% by weight at the expense of cellulose, while also adjusting a 50/50 blend of aspartate and glutamate to maintain the diets

isonitrogenous (Table 10). A fourth diet substituted the 50/50 blend of aspartate and glutamate with additional cellulose (HiCel). Each diet was fed to fish in four replicate aquaria.

The salinity of the culture system for the first 7 weeks of the trial was maintained at 3 g/L by mixing artificial sea salts with well water. After 7 weeks, the fish were weighed and sampled. Then, culture system salinity was increase to 15 g/L over 4 days and the trial continued for an additional 31 days (total of 5 weeks) with triplicate aquaria assigned to juvenile HSB fed the basal and creatine-supplemented diets at 1, 2, and 4% of dry weight. Each aquarium had 12 fish with an individual initial average weight of 27.6 g/fish, 28.6 g/fish, 29.8 g/fish, and 29.6 g/fish for the basal, 1%, 2%, and 4% diets, respectively. To compare effects of duration of creatine supplementation, 40 fish previously fed the HiCel diet were implanted with passively integrated transponder (PIT) tags and placed in four aquaria within the same recirculating system operating at 15 g/L. Each aquarium had 10 fish with an individual initial average weight of 29.5 g/fish, 28.7 g/fish, 30.2 g/fish, and 30.1 g/fish and were randomly assigned to either the basal, or creatine-supplemented diets at 1, 2, and 4% by weight, respectively.

Fish were initially fed 7% of their body weight during the low-salinity portion of the trial, and initially fed 4% of body weight during the brackish water portion of the trial. Daily rations were divided into two feedings per day, and each aquarium of fish was collectively weighed weekly at which time feed rations were adjusted to maintain a rate close to apparent satiation without overfeeding.

At the end of the two phases of the trial, fish were weighed collectively from each aquarium and tagged fish were weighed individually. Three fish from each tank were euthanized and subsequently homogenized to determine whole-body proximate composition according to established procedures (Brown *et al.* 1992). Three additional fish were anesthetized with tricaine

Table 10. Composition of experimental diets (g per 100 g dry weight).

	Diet designation/Creatine supplement (%)				
Ingredient	Basal	HiCel	1	2	4
Menhaden fishmeal	29.00	29.00	29.00	29.00	29.00
Soy protein conc.	28.00	28.00	28.00	28.00	28.00
Dex. Starch	20.00	20.00	20.00	20.00	20.00
Menhaden oil	6.31	6.31	6.31	6.31	6.31
Vitamin premix	3.00	3.00	3.00	3.00	3.00
Mineral premix	4.00	4.00	4.00	4.00	4.00
Carboxymethyl Cellulose	2.00	2.00	2.00	2.00	2.00
Aspartate/Glutamate	4.00	0.00	3.00	2.00	0.00
Creatine	0.00	0.00	1.00	2.00	4.00
Celufil	3.69	7.69	3.69	3.69	3.69
Analyzed proximate Composition [1]					
Dry matter	89.1	88.9	89.8	89.6	90.3
crude protein	39.8	39.5	40.3	40.5	41.2
crude lipid	10.3	10.4	10.2	10.4	10.3
Ash	6.5	6.4	6.7	6.5	6.6
Creatine	0.1	0.1	1.2	1.9	3.8

[1]Values are means of duplication analyses.

methane sulfonate (MS-222, 150 mg L^{-1}), weighed and dissected to obtain liver and intraperitoneal fat (IPF) for computing hepatosomatic index (HSI), and IPF ratio, respectively. Muscle tissue samples also were obtained for determination of proximate composition and creatine content. Creatine was analyzed in diets and muscle tissues using the Jaffe reaction as described by (Bonsnes and Taussky 1945). The responses utilized to compare treatment effects in this study were calculated as follow:

Weight gain, % = [(Final weight – initial weight)/(initial weight)] × 100;

Feed efficiency ratio (FE) = [weight gain (g)/dry feed consumed (g)];

Survival = [initial # of fish/ final # of fish] x 100;

Viscerosomatic indices (HSI, IPF ratio or fillet ratio, %) = [(liver, IPF, or fillet weight, g)/(BW, g)] ×100.

All data were analyzed for homogeneity of variances (Levene's test). The weight gain, feed efficiency and mortality responses of fish in the feeding trials were analyzed using one-way analysis of variance with significance level set at 5% using the Statistical Analysis System (version 9.0, SAS Institute, Cary, NC, USA).

IV.3 Results

Hybrid striped bass grew rapidly during the low-salinity phase of the feeding trial with fish fed all dietary treatments gaining over 1000% of their initial weight but showing no significant differences due to supplementation of creatine or additional cellulose to the basal diet (Table 11). However, during the brackish-water phase of the trial, significant differences in weight gain were found between fish fed the diets supplemented with creatine at 2% and 4% compared to those fed the basal and 1% creatine diets (Table 12). The weight gain response of

fish fed all diets in this second phase was lower than the first phase due to the larger initial weight of the fish and shorter duration. Feed efficiency also demonstrated no differences among dietary treatments during the low-salinity phase, but tended (P = 0.098) towards significance in the brackish-water phase with fish fed the creatine-supplemented diets having higher values (Tables 11 and 12). Survival, and condition indices including HSI, IPF, and fillet ratio showed no significant differences in either phase of the feeding trial (Tables 11 and 12).

A significant increase was detected in the weight gain of tagged fish fed the diet supplemented with creatine at 4% compared to those fed the basal diet (Table 13). There also were no significant differences in HSI, IPF, or fillet ratio of fish fed the various diets. Results for tagged fish were similar to non-tagged fish indicating no negative effects of tagging.

Whole-body composition of fish during the low-salinity and brackish water phases showed no significant differences in moisture, crude protein, crude lipid, or ash content regardless of diet (Table 14). Muscle composition of HSB fed the creatine-supplemented diets showed a slight but significant increase in crude protein content during the low-salinity phase of the trial, and a similar trend (P = 0.052) was noted during the brackish-water phase (Table 15). These differences were likely attributed to the high nitrogen content of creatine which was significantly elevated in the muscle tissue of fish fed the creatine-supplemented diets. Tagged fish in the brackish-water phase of the feeding trial did not demonstrate increased protein in muscle tissue, and though there was a significant difference in muscle creatine between tagged fish fed the basal diet compared to those fed the 4% creatine diet, muscle creatine in fish fed the other supplemented diets were not statistically distinct (Table 16). There were no significant differences in muscle moisture, lipid or ash composition between tagged or non-tagged fish.

Table 11. Growth performance of hybrid striped bass after 7 weeks of the low-salinity phase of the feeding trial.[1]

Response	Diet designation /Creatine supplement						
	Basal	HiCel	1%	2%	4%	P-Value	Pooled Std Error
Initial weight (g)	2.2	2.1	2.2	2.3	2.2	0.6668	0.06648
Weight gain (% of initial weight)	1109	1146	1087	1113	1151	0.7297	37.421
Survival (%)	99	96	100	99	96	0.4554	1.8976
Feed efficiency (g gain/g fed)	0.8	0.78	0.77	0.75	0.79	0.5036	0.02033
Hepatosomatic index (%)	2.52	2.63	2.44	2.57	2.51	0.7156	0.09377
Intraperitoneal fat ratio (%)	4.22	4.05	3.95	4.19	4.1	0.7045	0.14581
Fillet ratio (%)	37.8	37.3	37.5	37.6	37.4	0.7014	0.25314

[1] Values are means of four replicate groups.

Table 12. Growth performance of hybrid striped bass after 5 weeks of the brackish-water phase of the feeding trial.[1]

Response	Diet designation /Creatine supplement					
	Basal	1%	2%	4%	P-Value	Pooled Std Error
Initial weight (g)	27.6	28.6	29.8	29.6	0.232	0.78274
Weight gain (% of initial weight)	72[b]	74[b]	85[a]	83[a]	0.0449	3.1503
Survival (%)	94	92	94	86	0.33	3.4021
Feed efficiency (g gain/g fed)	0.62	0.63	0.71	0.68	0.0983	0.2508
Hepatosomatic index (%)	2.64	2.65	2.71	2.55	0.8392	0.11986
Intraperitoneal fat ratio (%)	4.93	5.1	4.73	4.85	0.8666	0.34521
Fillet ratio (%)	38.78	40.47	40.32	41.18	0.1456	0.65603

[1] Values are means of three replicate groups.

Table 13. Growth performance of tagged hybrid striped bass after 5 weeks of the brackish-water phase of the feeding trial.[1]

Response	Diet designation /Creatine supplement					
	Basal	1%	2%	4%	P-Value	Pooled Std Error
Initial weight	29.5	28.7	30.2	30.1	0.8066	1.8075
Weight gain (% of initial weight)	71[b]	79[ab]	83[ab]	89[a]	0.0375	4.1768
Survival (%)	90	100	100	100	NA	NA
Feed efficiency (g gain/g fed)	0.66	0.67	0.69	0.75	NA	NA
Hepatosomatic index (%)	2.5	2.6	2.7	2.6	0.1242	0.0547
Intraperitoneal fat ratio (%)	4.8	4.7	4.6	4.7	0.8929	0.12242
Fillet ratio (%)	40	41	41	41	0.5976	0.39705

[1]Values are means of 10 fish.

Table 14. Whole-body composition (% of fresh weight) of hybrid striped bass after 7 weeks of the low-salinity phase and 5 weeks of the brackish-water phase of the feeding trial.

	Diet designation /Creatine supplement						
	Basal	HiCel	1%	2%	4%	P-Value	Pooled Std Error
Low Salinity[1]							
Moisture	66.8	66.9	67.3	67	67	0.1285	0.13261
Crude Protein	19.0	18.9	18.7	18.9	18.7	0.6181	0.15733
Crude Lipid	9.6	10.2	9.5	9.1	9.8	0.7917	0.43562
Ash	4.8	4.8	4.6	4.5	4.7	0.806	0.17334
Brackish [2]							
Moisture	66.4	-	66.4	66	65.9	0.3092	0.21944
Crude Protein	18	-	18.2	17.9	18.1	0.8464	0.22582
Crude Lipid	12.3	-	12.4	12.1	12.5	0.2176	0.1178
Ash	4.4	-	4.5	4.5	4.5	0.9205	0.17753

[1]Values are means of composite samples from four replicate groups.
[2]Values are means of composite samples from three replicate groups.

Table 15. Muscle composition (% of fresh weight) of hybrid striped bass after 7 weeks of the low-salinity phase and 5 weeks of the brackish-water phase of the feeding trial.

	Diet designation /Creatine supplement						
	Basal	HiCel	1%	2%	4%	P-Value	Pooled Std Error
Low Salinity[1]							
Moisture	75.1	75.1	74.9	74.8	74.7	0.9133	0.09076
Crude protein	19.2[b]	19.2[b]	19.3[ab]	19.3[ab]	19.4[a]	0.0083	0.02467
Crude lipid	4.8	4.4	4.4	4.6	4.4	0.4401	0.14747
Ash	1.6	1.6	1.6	1.6	1.6	0.7676	0.04271
Creatine	0.37[b]	0.36[b]	0.43[a]	0.44[a]	0.44[a]	0.0005	0.01276
Brackish [2]							
Moisture	74.8	-	75.1	74.6	74.4	0.6432	0.12761
Crude protein	19.2	-	19.27	19.36	19.35	0.0523	0.3371
Crude lipid	4.96	-	4.72	4.69	4.81	0.2081	0.08819
Ash	1.47	-	1.45	1.48	1.47	0.8807	0.02667
Creatine	0.37[b]	-	0.45[a]	0.43[a]	0.44[a]	0.0196	0.01491

[1]Values are means of composite samples from four replicate groups.
[2]Values are means of composite samples from three replicate groups.

Table 16. Muscle composition of tagged hybrid striped bass after 5 weeks of the brackish-water phase of the feeding trial.[1]

	Diet designation /Creatine supplement					
	Basal	1%	2%	4%	P-Value	Pooled Std Error
Moisture	24.9	25.5	25.1	25	0.1802	0.17393
Crude Protein	19.2	19.3	19.3	19.3	0.4829	0.02743
Crude Lipid	4.9	4.8	4.9	4.8	0.9613	0.06158
Ash	1.6	1.5	1.5	1.5	0.0895	0.2243
Creatine	0.36[b]	0.39[ab]	0.39[ab]	0.41[a]	0.0368	0.01085

[1]Values are means of 10 fish.

Non-tagged fish fed basal and creatine-supplemented diets for a total of 12 weeks showed no difference in weight gain during the 5-week brackish-water phase of the trial compared to tagged fish fed the various diets. However, muscle creatine concentration was numerically higher in non-tagged fish fed creatine-supplemented diets throughout both phases of the trial compared to tagged fish fed the diets for only 5 weeks, and significantly so in those fed the diet with 1% supplemental creatine (Table 17).

IV.4 Discussion

Growth Performance

Creatine supplementation had no effect on growth, survival, or feed efficiency of HSB in freshwater, and thus does not appear to be required for improved growth performance. In brackish water, however, supplemented creatine improved growth performance which likely was attributed to assistance with osmoregulation through increased energetic efficiency. Supplementing the basal diet with a 50/50 blend of aspartate and glutamate, two non-essential amino acids, which could potentially be used as energy substrates (Wu *et al.* 2015) did not appear to provide increased metabolic energy compared to the creatine-supplemented diets in brackish water, but rather provided similar performance as fish fed the HiCel basal diet. These results are consistent with previous trials in which an excess of dietary aspartate and glutamate did not significantly improve weight gain of HSB in low-salinity water (Wu *et al.* 2015).

Table 17. Comparison of weight gain and muscle creatine concentrations of non-tagged hybrid striped bass vs tagged hybrid striped bass fed the basal and creatine-supplemented diets during the brackish-water phase of the feeding trial. Non-tagged fish had been fed the experimental diets for a total of 12 weeks compared to the tagged fish that were fed those diets for 5 weeks.

Response	Diet designation /Creatine supplement			
	Basal	1%	2%	4%
Weight gain (tagged)[1]	71.4	78.5	82.5	89
Weight gain (non-tagged)[2]	71.5	74.1	84.6	83.2
P-Value	0.9883	0.5755	0.7655	0.4675
Pooled Std Error	7.3013	6.4418	5.8887	6.7776
Creatine (tagged)[2]	0.36	0.39	0.39	0.41
Creatine (non-tagged)[2]	0.37	0.45	0.43	0.44
P-Value	0.4971	0.0109	0.1088	0.2345
Pooled Std Error	0.01033	0.01308	0.1648	0.01352

[1]Values are means of 10 fish.
[2]Values are means of three replicate groups.

Creatine

Supplementing the diet with creatine not only increased growth performance of HSB in brackish water, but also increased muscle creatine content in both low-salinity and brackish-water phases of the current study. Fish supplemented with creatine for the entire duration of the trial had higher creatine levels than the tagged fish fed the creatine-supplemented diets for only 5 weeks. Muscle creatine levels increased with the supplementation of creatine, but, for fish, the process of maximizing creatine muscle concentration appears to be slower than for humans (Danulat and Hochachka 1989, McFarlane *et al.* 2001). In humans, creatine is often supplemented in a loading phase of 20 g/d for 5-6 days then 2-5 g/d to maintain saturation (Hultman *et al.* 1996). McFarlane *et al.* (2001) found that after 7 days, muscle creatine levels in rainbow trout did not increase further. After 5 weeks of feeding in the brackish-water phase of the current study, dietary creatine supplementation at 2% by weight tended to raise muscle creatine levels of hybrid striped bass compared to those fed the basal diet, but not significantly as seen in fish fed the 4% supplemental level. Whether creatine concentration in muscle is reduced in fish cultured in brackish water due to increased creatine utilization in the gills (Danulat and Hochachka 1989) or duration of supplementation (McFarlane *et al.* 2001) remains to be determined. However, this study highlights a mechanism for HSB to transport and store creatine in its muscle tissue. Vascular injected creatine in flounder was reported to be taken up by muscle tissue (Danulat and Hochachka 1989).

Muscle creatine levels of HSB ranged from 0.36% to 0.45% representing a baseline and saturation, respectively. Augmenting muscle creatine concentration seems slow, as 5 weeks feeding of the diet with 2% supplementation did not significantly increase muscle creatine concentration as it did at 4% supplementation. There is potential for diets with greater than 4%

inclusion of creatine to more rapidly increase muscle creatine concentration. However, this study is the first to measure creatine concentrations in HSB and indicate their levels to be similar to several teleost species. Ergogenic benefits of increased muscle creatine storage in mammals are improved with sustained supplementation (Hultman *et al.* 1996), yet, there has been no clear understanding of long-term vs short-term effects of creatine in fish.

Salinity Change

Fishes can gradually adapt to osmotically challenging environments (Kilambi and Zdinak 1980, Weirich and Tomasso 1991, Molina *et al.* 2016). Hybrid striped bass have been acclimated to various salinities at a rate between 3-5 g/L/d without any ill effects (Weirich and Tomasso 1991, Riche 2007). In this study, salinity was increased from 3 g/L to 15 g/L over 4 days (3.25 ppt/d). After the change in salinity, stress became evident in fish fed all diets as weight gain and feeding rates decreased. There was no apparent effect of added stress due to tagging the fish that were individually monitored in this phase of the feeding trial.

Nutritional studies with fishes comparing growth performance in response to a second environmental variable such as temperature (Woiwode and Adelman 1991, Wyban *et al.* 1995, Amin *et al.* 2016) or salinity (Brown *et al.* 1992, Gatlin *et al.* 1992), typically utilize multiple groups of fish raised in separate systems simultaneously or the same system with different fish cultured at different times. While some of these studies have proven effective in determining interactions between variables (Brown *et al.* 1992, Gatlin *et al.* 1992, Cabanillas-Beltran *et al.* 2001, Lim *et al.* 2006) it would be optimal to examine nutritional and environmental variables at the same time. In regards to the current study, when salinity was increased at the end of week 7, some fish previously fed the HiCel diet in low-salinity water and thus not previously exposed to

supplemental creatine were PIT-tagged then fed the basal and creatine-supplemented diets for the duration of the brackish water phase. This allowed a comparison of fish fed the basal and creatine-supplemented diets for either 5 weeks or 12 weeks.

Growth performance of non-tagged and tagged fish raised in brackish water were similar in this study, indicating that pre-loaded creatine concentration have no bearing on improving osmoregulation in hypersaline environments for hybrid striped bass. Osmoregulation is primarily in the gills and intestine of fish (Gatlin *et al.* 1992, Jackson *et al.* 2005). The creatine - creatine phosphate shuttle, where creatine acts as a regenerative source of phosphate for ATP, has been recognized in the gills of fish (Kultz and Somero 1995, Lin *et al.* 2003). Thus, increased circulating creatine could be taken up by gills, and energetically assist in osmoregulation.

Conclusions

This study examined the effect of dietary creatine supplementation in low-salinity and brackish waters, and a temporal effect of creatine supplementation in brackish water. Creatine supplementation was beneficial to HSB in brackish water, but had no effect in low-salinity water. Fish fed diets supplemented with creatine for the entire 12 weeks maximized muscle creatine concentrations at 0.45% of fresh weight, but duration of supplementation had no effect on growth performance of HSB in brackish water. Increased muscle creatine concentration from 0.36% to 0.45% of fresh weight indicated a mechanism for creatine uptake and may represent muscle creatine saturation in HSB.

CHAPTER V

EFFECTS OF DIETARY CREATINE ON RED DRUM JUVENILES IN WATER OF VARIOUS SALINITIES

V.1 Introduction

The red drum is a carnivorous, euryhaline sciaenid cultured in the southern region of the United States, Mexico, Ecuador, and China. It is fast-growing species cultured for both food and stock enhancement to support recreational fishing. Popularity of red drum as a food fish and limited regulation of commercial fishing in the 1980s lead to overfishing and a decline in natural populations in the Gulf of Mexico, prompting a closure of the commercial fishery and accelerated efforts to culture the species (Gatlin 2002). A variety of research efforts have focused on improving the production of red drum, including refinement of diet formulations based on a greater understanding of the nutritional requirements of the species. The current study focused on evaluating the potential benefits of dietary creatine on red drum.

Fishmeal is the preferred protein feedstuff for carnivorous species, however, due to the expansion of world aquaculture, demand for fishmeal has dramatically increased, resulting in it becoming cost prohibitive. Thus, over the past decade research has focused on substituting fishmeal with other protein feedstuffs including animal by-products and plant proteins (Gatlin *et al.* 2007, Hardy 2010) These substitutes often do not have the optimal amino acid profile to sustain proper growth and health of carnivorous fishes (NRC 2011). Methionine (Mambrini *et al.* 1999, Goff and Gatlin 2004) and lysine (Craig and Gatlin 1992, Gaylord *et al.* 2004) are indispensable amino acids often limiting in plant proteins and thus must be supplemented in plant-based diets (Hardy 2010). Fishmeal also provides other organic acids such as creatine

(Ringel *et al.* 2008) and taurine (Hardy 2010) which are devoid in plant feedstuffs and might be required in the diet of carnivorous fishes fed plant-based diets.

Creatine is an organic acid commonly supplemented in the diets of humans (Harris *et al.* 1992) and mammals (Schoch *et al.* 2006, Carvalho *et al.* 2013, Janicki and Buzała 2013) to improve ergogenic and growth performance. There are several functions of creatine that have been documented in mammals: it serves as a cellular buffer (Newsholme and Beis 1996); improves neuromuscular control (Iqbal *et al.* 2015); conserves endogenous precursors (Wyss and Kaddurah-Daouk 2000, Ostojic *et al.* 2014); and increases protein synthesis (Kreider *et al.* 1998, Shankaran *et al.* 2016). In fish, creatine has the added potential benefit of assisting in osmoregulation due to the increase in creatine kinase activity in the gills during times of osmotic stress (Kultz and Somero 1995, Ching-Feng *et al.* 2002, Lin *et al.* 2003). Creatine primarily provides additional phosphorus, in the form of phosphocreatine (PCr), in muscle tissue. The inability to endogenously produce sufficient creatine in humans leads to mild to severe intellectual inabilities (Hanna-El-Daher and Braissant 2016). Thus, it is well established that creatine is necessary for normal animal development, health and survival. However, whether supplementation of creatine can improve some aspects of growth and metabolism has not been adequately addressed in cultured fishes.

In mammals, creatine is endogenously produced by enzymes in the kidney [arginine-glycine amidinotransferase (AGAT)] and liver [guanidinoacetate N-methyltransferase (GAMT)] from arginine, glycine, and methionine, then transported to muscle tissue by a creatine transporter (Schoch *et al.* 2006). In fish, creatine anabolizing enzymes are found in muscle tissue (Borchel *et al.* 2014) putting in question the transport mechanism of exogenous creatine into muscle tissue. Also, compared to mammals, creatine turnover is slower in fishes (Danulat and

Hochachka 1989). Thus, two separate feeding trials were conducted to examine the potential benefits of creatine supplementation in practical diets for red drum. In addition, this study examined if long-term creatine supplementation increased creatine content in red drum muscle tissue.

V.2 Materials and Methods

Two consecutive feeding trials were conducted to examine the effects of supplementing creatine to the diet on growth performance of red drum in low salinity (3 ppt, 5 ppt,) and moderate salinity (15 ppt) environments. Red drum for the two separate trials were transported from Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center operated by Texas Parks and Wildlife Department in Lake Jackson, TX and maintained indoors in closed recirculating water systems at the Aquacultural Research and Teaching Facility of the Texas A&M University System. Water temperature was maintained at 26 C by conditioning ambient air and dissolved oxygen was provided by a regenerative blower through air stones to provide levels close to air saturation. A 12h light: 12h dark photoperiod was maintained using fluorescent lighting controlled by timers.

Red drum were conditioned on a commercial diet containing 40% crude protein and 12% lipid (Rangen, Inc) and were acclimated for 1 week in 38-L aquaria for trial I and 110-L aquaria for trial II. Each system operated as independent recirculating systems with a settling chamber, submerged media biofilter, ultraviolet light sterilizer and sand filter. For trial I, each aquarium was stocked with 11 fish with an initial average weight of 8.9 ± 1.8 g/fish and salinity was maintained at 5 ppt. For trial II, each aquarium was stocked with 15 fish with an initial average weight of 7.3 ± 1.5 g/fish. The salinity for trial II was maintained at 3 ppt for 7 weeks. Then,

after 7 weeks, system salinity was gradually increased to 15 ppt over 4 days and the trial continued for an additional 31 days (total of 5 weeks).

The basal diet for both trials was formulated to contain 40% crude protein, contributed equally by menhaden fishmeal and soy protein concentrate, and 10% lipid (Table 18). For trial I, five experimental diets were derived from the basal diet by supplementing creatine at 0.5, 1, 1.5, 2, or 4% by weight at the expense of cellulose. The diets for trial I were adjusted with a 50/50 blend of aspartate and glutamate to maintain the diets isonitrogenous. For trial II, the same basal diet and one supplemented with creatine at 2% by weight were prepared with the creatine-supplemented diet adjusted with a 50/50 blend of aspartate and glutamate on an equal-weight basis (Table 19).

Fish in both trials were initially fed at 5% of their body weight per day. The daily feed allowance was divided into morning and evening feedings, and each aquarium of fish was group weighed weekly to adjust feed rations. By the end of each trial, fish were fed 3% of their body weight.

Sampling and analyses

At the end of each trial, three fish from each aquarium were anesthetized with tricaine methane sulfonate (MS-222, 150 mg L^{-1}), weighed and dissected to obtain liver, muscle and intraperitoneal fat (IPF) for computing hepatosomatic index (HSI) ([liver weight * 100]/whole-body weight), muscle ratio ([fillets weight*100]/whole-body weight) and IPF ratio ([IPF weight * 100]/whole-body weight). Three additional fish per aquarium were sampled and homogenized for whole-body proximate composition. Muscle tissue samples also were obtained from three

Table 18. Composition (g per 100 g dry weight) of experimental diets fed to juvenile red drum in trial I.

	Diet designation/Creatine supplement (%)					
Ingredient	0	0.5	1	1.5	2	4
Menhaden Fishmeal	29.0	29.0	29.0	29.0	29.0	29.0
Soy Protein Conc.	28.0	28.0	28.0	28.0	28.0	28.0
Dextrinized Starch	20.0	20.0	20.0	20.0	20.0	20.0
Menhaden Oil	6.1	6.1	6.1	6.1	6.1	6.1
Vitamin Premix	3.0	3.0	3.0	3.00	3.00	3.0
Mineral Premix	4.0	4.0	4.0	4.00	4.00	4.0
Carboxymethyl Cellulose	2.00	2.0	2.0	2.00	2.00	2.0
Aspartate/Glutamate	6.4	4.8	3.2	1.6	-	-
Creatine	-	0.5	1.0	1.5	2.0	4.0
Celufil	1.5	2.6	3.7	4.8	5.9	3.9
Analyzed proximate composition (% dry weight)[1]						
Dry matter	87.9	89.1	89.8	86.8	89.2	88.3
Crude protein	41.2	40.8	40.2	40.3	41.4	40.9
Crude lipid	10.4	10.2	10.3	10.1	10.2	10.1
Ash	6.3	6.4	6.6	6.5	6.8	6.7
Creatine	0.09	0.55	1.13	1.54	2.08	3.78

[1] Values are means of duplicate analyses.

Table 19. Composition (g per 100 g dry weight) of experimental diets fed to juvenile red drum in trial II.

	Diet	
Ingredient	Basal	2% creatine
Menhaden Fishmeal	29.00	29.00
Soy Protein Conc.	28.00	28.00
Dex. Starch	20.00	20.00
Menhaden Oil	6.31	6.31
Vitamin Premix	3.00	3.00
Mineral Premix	4.00	4.00
Carboxymethyl Cellulose	2.00	2.00
Aspartate/Glutamate	4.00	2.00
Creatine	-	2.00
Celufil	3.69	3.69
Analyzed proximate Composition[1]		
Dry matter	89.1	89.6
crude protein	39.8	40.5
crude lipid	10.3	10.4
Ash	6.5	6.5
Creatine	0.1	1.9

[1] Values are means of duplicate analyses.

additional fish per aquarium for analysis of proximate composition and creatine content. Analyses for proximate composition were as previously described (Rosales *et al.* 2017). Creatine was analyzed in diets and muscle tissues using the Jaffe reaction as described by Bonsnes and Taussky (1945).

Statistical analyses

Responses of fish in trials I and II were subjected to analysis of variance using the Statistical Analysis System (version 9.4, SAS Institute, Cary, NC, USA). Responses for trial I were also analyzed by unequal polynomial regression analysis to detect potential linear or quadratic effects (Robson 1959, Davis 2010). The minimum dietary creatine requirement for optimal weight gain was estimated with broken-line regression. If there were significant differences in trial II, means were subjected to Tukey's test. Significance level was set at 5% for all analyses.

V.3 Results

Juvenile red drum fed diets supplemented with creatine had a significant positive linear and quadratic trend in percent weight gain, survival, and feed efficiency in trial I (Table 20). Broken-line regression analysis of weight gain data indicated the minimum creatine supplementation level for optimal weight gain to be 1.98% of diet (Fig. 1). During the low-salinity and moderate-salinity periods of trial II, there was a numerical increase and a trending (P=0.057) increase in percent weight gain of fish fed the creatine-supplemented diet, respectively (Table 21). Furthermore, percent weight gain for the entirety of trial II was significantly improved for red drum fed the creatine-supplemented diet. There was no significant difference in

survival of red drum in trial II, but fish fed the creatine-supplemented diet did tend to exhibit improved feed efficiency in the moderate-salinity portion of the trial (P=0.081) and for the entire trial (P=0.063).

There was a linear increasing trend in HSI of fish fed creatine-supplemented diets in trial V (Table 20), and trial II showed a numerical increase in HSI with creatine supplementation, though not significant (Table 21). There were no significant differences attributable to diet with regard to IPF ratio or muscle ratio in either trial. There was no significant difference in dietary creatine supplementation on whole-body dry matter, protein, lipid or ash in either trial (Tables 22 and 23).

Muscle composition of red drum showed a significant linear trend in muscle protein percentage in response to creatine supplementation in trial I (Table 22), and a significant difference in trial II (Table 23). This can potentially be attributed to supplementation of all levels of creatine having significantly more creatine (a highly nitrogenous compound) in muscle tissue. Muscle creatine had a positive linear and quadratic trend in fish fed the creatine-supplemented diets (Table 22). There was no significant difference in dietary creatine supplementation on muscle moisture, lipid or ash in either trial.

V.4 Discussion

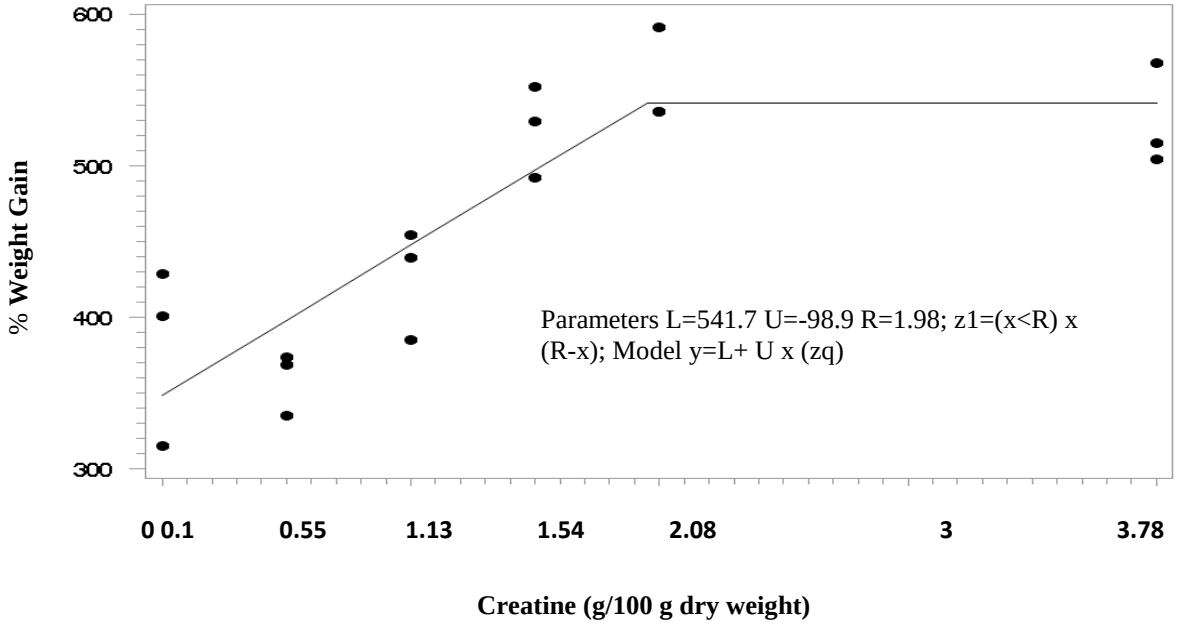
Fishmeal modestly contributes creatine when included in diets of aquatic species. But due to increasing demand and consequently escalated price of fishmeal, considerable efforts have been made to replace fishmeal with various alternative protein feedstuffs. Various plant protein concentrates can contribute a considerable amount of dietary protein for red drum (Rossi Jr *et al.* 2013) but are devoid of creatine. Natural diets and possibly endogenous creatine

Table 20. Growth performance of juvenile red drum fed graded levels of creatine in trial I.

Response	Diet designation/Creatine supplementation (%)[1]								
	0	0.5	1	1.5	2	4	ANOVA[2]	Linear[2] Trend	Quadratic[2] Trend
Initial Weight (g)	8.9	8.9	8.9	8.8	8.8	8.9			
Weight Gain (% of initial weight)	382^{c}	360^{c}	426^{bc}	524^{ab}	554^{a}	529^{a}	0.00	0.0001	0.001
Survival, %	59^{c}	71^{bc}	86^{ab}	86^{ab}	86^{ab}	92^{a}	0.00	0.0001	0.0013
Feed Efficiency (g gain/g fed)	0.44^{b}	0.51^{b}	0.68^{a}	0.78^{a}	0.81^{a}	0.82^{a}	0.00	0.0001	0.0001
Muscle Ratio, %	27.8	28	27.6	28.1	27.9	28	0.15	1	0.6879
Hepatosomatic Index (%)	2.0^{c}	2.8^{ab}	2.3^{bc}	2.4^{bc}	2.54$^{a}_{b}$	3.1^{a}	0.00	0.0002	0.7484
Intraperitoneal Fat Ratio (%)	0.54	0.66	0.55	0.57	0.63	0.56	0.88	0.8397	0.6873

[1] Values are means of three replicate groups.
[2](Pr > F).

Figure 1 **Broken-line regression of weight gain against dietary creatine supplementation.**



Relationship between weight gain of juvenile red drum and supplemental creatine level as described by a broken-line regression model. The break point estimate is interpreted as the creatine level above which there is no significant change in the response criteria. Each dot in the curve represents the average weight gain of fish in each experimental unit (triplicate aquaria per treatment). Parameters are defined as L = plateau, U = slope and R = breaking point.

Table 21. Growth performance of juvenile red drum fed a basal or creatine-supplemented diet in low-salinity (LS) and moderate salinity (MS) environments in trial II.

Response	Dietary creatine supplementation (%)			
	0	2	P-Value	Pooled Std error
Initial Weight (LS)	7.2	7.4	0.394	0.16043
Initial Weight (MS)	33.4	35.3	0.383	1.3624
Weight Gain (LS) %	349.8	390.4	0.156	16.464
Weight Gain (MS) %	48.8	75.9	0.057	7.241
Weight Gain (Entire trial) %	570	762.6*	0.040	45.45
Survival (LS)%	73	73	1	0.12472
Survival (MS)%	97	97	0.960	2.4734
Survival (Entire trial)%	71	71	1	0.11111
Feed Efficiency (LS)	0.92	0.97	0.312	0.03466
Feed Efficiency (MS)	0.49	0.66	0.081	0.05455
Feed Efficiency (Entire trial)	0.78	0.90	0.063	0.03182
Hepatosomatic Index, %	2.63	2.76	0.416	0.09369
Intraperitoneal Fat Ratio %	0.37	0.32	0.460	0.04011
Muscle Ratio, %	27.5	27.8	0.361	0.22436

Final weight for LS fish was 49.4 g/fish and 62.1 g/fish for the MS phase. There was a significant difference amongst treatments (ANOVA P = 0.046).

* Denotes significant ($P \leq 0.05$) difference.

Table 22. Proximate composition of whole-body and muscle tissues of red drum fed diets with graded levels of creatine in trial I.

	Creatine supplementation (%)[1]								
	0	0.5%	1%	1.5%	2%	4%	ANOVA[2]	Linear[2] Trend	Quadratic[2] Trend
Whole-Body									
Moisture (%)	72.4	73.2	74.1	71.8	72.6	72.5	0.15	0.5197	0.9192
Protein (%)	17.8	17.5	17.6	17	17.2	17.6	0.21	0.5672	0.0413
Lipid (%)	6.4	6.9	7.2	6.8	7.2	7	0.27	0.1924	0.1311
Ash (%)	3.42	3.37	3.58	3.49	3.5	3.48	0.33	0.7361	0.3505
Muscle									
Moisture (%)	75.4	74.9	74.9	75.2	74.8	75.3	0.31	0.9737	0.1319
Protein (%)	21.3	22.5	22.6	22.2	22.7	22.9	0.07	0.0228	0.1651
Lipid (%)	1.24	1.31	1.26	1.24	1.3	1.29	0.11	0.1396	0.958
Creatine (%)	0.31[b]	0.39[a]	0.43[a]	0.4[a]	0.43[a]	0.4[a]	0.00	0.0017	0.0001

[1] Values are means of duplicate replications.
[2](Pr > F).

Table 23. Body condition indices and whole-body composition of red drum in trial II after 5 weeks of being cultured at the moderate salinity.[1]

	Diet			
	Basal	2%	P-Value	STD Error
Whole-body				
Moisture (%)	72.6	72.4	0.2335	0.05548
Protein (%)	17.2	17	0.3653	0.15246
Lipid (%)	7.47	7.53	0.653	0.09232
Ash (%)	3.54	3.52	0.9274	0.9715
Muscle				
Dry Matter (%)	75.5	75.3	0.014	0.02472
Protein (%)	21.9	22.5*	0.049	0.14256
Lipid (%)	1.40	1.35	0.130	0.02108
Ash (%)	1.36	1.39	0.696	0.05617
Creatine (%)	0.32	0.42*	0.000	0.00745

[1] Values are means of duplicate analyses.

* Denotes significant ($P \leq 0.05$) difference.

synthesis (Wang *et al.* 2010, Borchel *et al.* 2014) adequately satisfies the creatine requirements of aquatic species. However, as plant protein feedstuffs increasingly replace fishmeal in the diet of carnivorous fish species, there may be greater need for dietary supplementation of creatine. The results of the two feeding trial in this study confirm that dietary supplementation of creatine improves growth performance of red drum in various osmotic environments. Requirements of amino acid metabolites, such as creatine, should be evaluated in other fish species as further replacement of fishmeal is pursued.

Creatine statistically improved weight gain of red drum in trial I (5 ppt), but only numerically improved weight gain during the low-salinity (3 ppt) phase in trial II. Furthermore, when salinity was increased in trial II to 15 ppt, a trend for increased weight gain with dietary creatine supplementation was detected. This trend in enhanced weight gain during 5 weeks of feeding in a moderate salinity environment suggests a potential effect of salinity on creatine requirements. Therefore, additional experiments will be pursued to further evaluate this potential effect of salinity on dietary creatine needs.

Optimal creatine supplementation of the diet for red drum in trial I was 1g creatine/kg of body weight based on their initial body weight. In addition, higher levels of dietary creatine showed no deleterious effects on red drum in the current study. Supplementation of creatine in humans is frequently delivered in a loading phase of 20g/d for 5-6 days then 2-5g/d in a liquid to maintain saturation (Hultman *et al.* 1996). For a 68 kg male, supplemented creatine during the loading phase is approximately 0.3 g/kg of body weight.

Relatively high mortality of red drum fed the basal diet in trial I compared to trial II is unclear; however, improvement in survival of fish fed the creatine-supplemented diets in trial I suggest creatine may also increase robustness of juvenile red drum. Creatine kinase is highly

active in early stages of fish development (Shaklee and Whitt 1977, Boulekbache 1981, Slenzka *et al.* 1993). This can be especially important for marine fishes as they are less developed and more fragile in early life stages than most freshwater species. To our knowledge there is no research concerning evaluation of dietary creatine effects on immune responses or development processes of fishes.

The HSI was generally higher in red drum fed diets with supplemental creatine in the current study. In the feeding trial of (Craig *et al.* 1995), red drum raised in full strength sea water (32 ppt) had greater HSI than red drum raised in brackish water (5 ppt). Dietary lipid also has significant impacts on HSI of red drum which store high levels of lipid in their liver (Serrano *et al.* 1992). For other fish species, elevated dietary carbohydrate can result in liver glycogen accumulation which can increase HSI (Shimeno 1979, Hilton and Atkinson 1982, Wu *et al.* 2015). However, the same level of soluble carbohydrate was included in the various diets fed to red drum in the current study. There is evidence for supplemental dietary nitrogenous compounds such as creatine to impact HSI ratios as seen in cobia (Lunger *et al.* 2007) and red drum (Pewitt *et al.* 2017) (Castillo and Gatlin 2018).

Muscle creatine levels of red drum ranged from 0.31 to 0.43% of fresh weight, representing baseline and saturation levels, respectively. An increase in muscle creatine indicates a mechanism for red drum to deposit creatine in muscle tissue. Muscle creatine in rainbow trout did not increase after 7 days of supplementation, but did increase muscle glycogen and improve sprint performance (McFarlane *et al.* 2001). The ability for fishes to deposit exogenous creatine may require longer periods of supplementation, or be species specific.

CHAPTER VI

EFFECTS OF DIETARY CREATINE ON RED DRUM JUVENILES CULTURED IN HYPOSMOTIC AND ISOSMOTIC WATERS

VI.1 Introduction

Creatine is an organic acid supplemented in the diets of humans (Harris *et al.* 1992) and mammals (Schoch *et al.* 2006, Janicki and Buzała 2013) to enhance (Carvalho *et al.* 2013) ergogenic and growth performance. Daily, creatine stores are lost due to spontaneous conversion to creatinine and are replenished via endogenous production or dietary supply (Wyss and Kaddurah-Daouk 2000). For example, humans lose approximately 2 grams of creatine per day and only endogenously produce 1 gram per day (Wyss and Kaddurah-Daouk 2000), leaving 1 gram to be provided by diet (Wyss and Kaddurah-Daouk 2000, Brosnan and Brosnan 2007). Creatine, in mammals is endogenously produced by two reactions. In the kidney, Arginine:glycine amidinotransferase attaches the amidino group of arginine to glycine, producing guanidinoacetic acid (GAA). Then GAA can be methylated by guanindinoacetate N-methyltransferase (GAMT), transferring a methyl group from methionine to produce creatine and S-adenosylhomocysteine. Exogenous creatine can be sourced from consumption of animal muscle tissue such as beef (Purchas *et al.* 2004) and fish (Brosnan *et al.* 2011).

Supplementing precursors of creatine synthesis has been examined in several animals. Arginine, a multifunctional essential amino acid, and guanidinoacetic acid (GAA), an amine arginine metabolite, are creatine precursors, and have been shown to increase serum and muscle creatine in broiler chickens given plant-based diets (DeGroot *et al.* 2017, Majdeddin *et al.* 2018). Dietary arginine and glycine increased creatine biosynthesis in chicks, rats and man (Walker

1979), but glycine alone did not increase muscle creatine in humans (Williams 1985). Supplemental arginine also improved muscle creatine in rats (Minuskin *et al.* 1981). In pigs GAA was effective in improving muscle creatine (McBreairty *et al.* 2015, He *et al.* 2018), and humans have recently began to develop protocols for supplemental GAA to increase endogenous creatine production (Ostojic *et al.* 2013). In red drum, supplemental arginine has been shown to improve immune response and intestinal structure (Cheng *et al.* 2011) but had limited effects on growth when supplemented above the minimum requirement (Fauzi 2013). There is little information on creatine metabolism for fishes, therefore, this study will examine supplemental arginine and glycine impact on muscle creatine.

Creatine functions as a regenerative energy source and storage of phosphorus for energy metabolism converting ADP to ATP. Creatine phosphate is catabolized and phosphate is liberated by creatine kinase (CK) in times of intense energy expenditure. Fish, similar to mammals, store 95% of creatine in the muscle tissue. Yet, isoforms of CK are also found in the brain (Slenzka *et al.* 1993), liver (Bankefors *et al.* 2011), testes (Greenwald 1946), kidney (Baldissera *et al.* 2017) and gills of fish (Lin *et al.* 2003). Activity of creatine kinase indicates the presence of a creatine-creatine phosphate shuttle that momentarily maintains ATP concentrations. In tilapia (*Oreochromis mossambicus*), CK activity increased in gills when placed in a hypersaline environment and helped maintain homeostasis (Ching-Feng *et al.* 2002). Euryhaline fish such as red drum are adept at osmoregulation in a wide range of water salinity.

Red drum is a euryhaline marine carnivorous species native to the Atlantic Ocean and Gulf of Mexico. They can be found in waters with salinity as high as 60 ppt, and have been cultured in freshwater, thus the effects of salinity on red drum has been the focus of several researchers (Crocker *et al.* 1983, Weirich and Tomasso 1991, Forsberg *et al.* 1996, Watson *et al.*

2014). To survive such a wide range of salinities, red drum can actively reverse the osmotic ion pump in their gills to maintain osmolality. Though osmoregulation requires energy, optimal growth of red drum was observed in a hyposmotic environment (Gatlin *et al.* 1992) and not in an isosmotic environment of 11 ppt (Wakeman and Wohlschlag 1985, Weirich and Tomasso 1991) as would be assumed. In general, growth of marine fishes is better at lower salinity (Boeuf and Payan 2001). Furthermore, various dietary requirements differ with salinity in several species (Zeitoun *et al.* 1973, Zeitoun *et al.* 1974, De Silva and Perera 1985, Brown *et al.* 1992). Gatlin *et al.* (1992) found adding sodium chloride to the diets of red drum was only significantly effective in freshwater for red drum. Knowing that the creatine-creatine phosphate shuttle is active in the gills of fish, there is potential for creatine to predominately function to energetically assist with osmoregulation and be ineffective in isosmotic conditions. Based on these various reasons, the present study examined growth performance of red drum fed supplemental creatine in hyposmotic and isosmotic environments.

VI. Materials and Methods

Two concurrent feeding trials were conducted to examine the effect of supplementing creatine to the diet on growth of red drum in hyposaline (6 ppt), and isosaline (11 ppt) environments. Red drum for the two separate trials were transported from Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center operated by Texas Parks and Wildlife Department in Lake Jackson, TX and maintained indoors in closed recirculating water systems at the Aquacultural Research and Teaching Facility of the Texas A&M University System. Water temperature was maintained at 26 C and dissolved oxygen maintained above acceptable levels

for red drum. A 12h light: 12h dark photoperiod was maintained using fluorescent lighting controlled by timers.

Red drum were conditioned on a commercial diet containing 40% crude protein and 12% lipid (Rangen, Inc) and were acclimated for 1 week in 38-L aquaria. Each system operated as independent recirculating systems with a settling chamber, submerged media biofilter and sand filter. In the two systems that were used, each aquarium was stocked with 15 fish with an average weight of 1.4 $\pm$ 0.6 g/fish. One of the systems was maintained at 6 ppt, while the other was 11 ppt by adjusting the concentration of synthetic sea water and NaCl mixed with fresh well water.

A basal diet was formulated to contain 40% crude protein, contributed equally by menhaden fishmeal and soy protein concentrate, and 10% lipid (Table 24). Six experimental diets were derived from the basal diet by supplementing incremental levels of creatine (1, 2 and 4%) at the expense of cellulose without adjusting their total nitrogen content. Another series of diets with creatine at 0, 1 and 2% were formulated to be isonitrogenous with a 50/50 arginine/glycine blend by weight. These diets were designated as Iso N.

Fish were initially fed at 6% of their body weight per day. The daily feed allowance was divided into morning and evening feedings, and each aquarium of fish was group weighed weekly to adjust feed rations. By the end of the trial, fish in both systems were consuming 4% of their body weight per day.

Samplings

At the end of each trial, three fish from each tank were anesthetized with tricaine methane sulfonate (MS-222, 150 mg L^{-1}) weighed and dissected to obtain liver, muscle and

Table 24. Composition (g per 100 g dry weight) of experimental diets fed to juvenile red drum.

	Diet designation/Creatine supplement (%)						
Ingredient	Basal	1	2	4	Basal Iso N	1 Iso N	2 Iso N
Menhaden Meal	29.0	29.0	29.0	29.0	29.0	29.0	29.0
Soy Protein Conc.	28.0	28.0	28.0	28.0	28.0	28.0	28.0
Dex. Starch	22.0	22.0	22.0	22.0	20.0	20.0	20.0
Menhaden Oil	6.3	6.3	6.3	6.3	6.3	6.3	6.3
Vitamin Premix	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mineral Premix	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Carboxymethyl Cellulose	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Creatine	-	1.0	2.0	4.0	-	1.0	2.0
Arginine/glycine	-	-	-	-	5.0	3.8	2.5
Celufil	5.7	4.7	3.7	1.7	0.7	0.9	1.2
Analyzed proximate Composition (% dry weight)[a]							
Dry matter	89.2	86.4	89.1	88.1	85.6	86	85.6
crude protein	40.8	41.2	41.4	43.8	41.6	41.7	42.5
crude lipid	10.2	9.9	10.3	10.6	10.2	10.4	10.3
Ash	6.6	6.4	6.5	6.7	6.7	6.4	6.7
Creatine	0.1	1.2	2.1	4.2	.04	1.1	2.3

[a]Values are means of duplicate analyses.

intraperitoneal fat (IPF) for computing hepatosomatic index (HSI) ([liver weight * 100]/whole-body weight), muscle ratio ([fillets weight*100]/whole-body weight) and IPF ratio ([IPF weight * 100]/whole-body weight). Three additional fish per aquarium were sampled and homogenized for whole-body proximate composition. Muscle tissue samples also were obtained from three additional fish per aquarium for analysis of proximate composition and creatine content. Analyses for proximate composition were as previously described (Rosales *et al.* 2017). Creatine was analyzed in diets and muscle tissues using the Jaffe reaction as described by (Bonsnes and Taussky 1945).

Calculations and Statistical Analyses

Responses were subjected to factorial analysis of variance using the Statistical Analysis System (version 9.0, SAS Institute, Cary, NC, USA) to determine potential interactions between dietary creatine level and water salinity. If there were no interactions, means were pooled and subjected to Tukey's test. Significance level was set at 5% for all analyses.

VI.3 Results

Growth Performance

Factorial analysis of variance revealed there were no interactions between water salinity and dietary creatine; therefore, data were pooled to discern any significant main effects (Table 25). Juvenile red drum fed the creatine-supplemented diets had significantly greater weight gain compared to those fed the basal diet. A main effect of salinity was determined to significantly increase survival of fish reared in isosmotic. Both main effects, salinity and diet, affected feed efficiency of red drum with fish in the hyposmotic environment having significantly higher

values for all diets. Supplementing diets with creatine improved feed efficiency compared to fish fed the basal diet except for fish fed the 1% creatine diet.

Some body condition indices were affected by the treatments with HSI being significantly lower in fish fed the isonitrogenous diets, and all creatine supplemented diets where numerically higher than non-supplemented diets. The IPF ratio values tended (P=0.06) to be different, and were significantly (P=0.03) affected by salinity and diet. Muscle ratio also was significantly affected with fish fed the diet with 2% creatine being significantly higher than those fed the isonitrogenous diets, and a trend (P=0.8) was detected with fish reared in isosmotic water having greater values than those on the hyposmotic water.

Whole-body Composition

There were no significant differences in whole-body composition of fish fed the various diets and reared in hyposmotic or isosmotic environments (Table 26). There was a salinity trend in whole- body lipid of fish raised in the isosmotic water which was numerically higher than hyposaline water.

Muscle Composition

There was no significant difference in muscle dry matter, lipid, or ash of red drum fed creatine-supplemented diets (Table 27). While muscle protein was numerically higher in fish fed the creatine-supplemented diets, there was no trend or significance. Creatine concentration increased in the muscle of fish fed the creatine-supplemented diets. Finally, the arginine/glycine supplement did not affect muscle creatine % as there was no difference in fish fed the basal diet vs the isonitrogenous diet without creatine.

Table 25. Growth performance of juvenile red drum fed diets with different levels of creatine in hypo- and isosaline environments.

Variable	Creatine %	[1]Weight gain %	Feed Efficiency	Survival %	Hepatosomatic index %	Intraperitoneal fat ratio %	Muscle ratio %
			Individual treatment				
Salinity							
Hyposaline	Basal	492^{bc}	0.73^{bcd}	84^{ab}	2.61^{ab}	0.22^{ab}	26
Isosaline	Basal	471^{c}	0.58^{d}	91^{a}	2.89^{ab}	0.16^{ab}	26
Hyposaline	1	573^{bc}	0.82^{abc}	71^{cd}	2.89^{ab}	0.11^{b}	26
Isosaline	1	572^{bc}	0.73^{bcd}	80^{bc}	2.71^{ab}	0.374^{ab}	27
Hyposaline	2	710^{abc}	0.83^{ab}	69^{cd}	3.22^{a}	0.27^{ab}	28
Isosaline	2	675^{abc}	0.74^{bc}	71^{cd}	2.86^{ab}	0.41^{a}	28
Hyposaline	4	709^{abc}	0.86^{ab}	73^{c}	3.13^{a}	0.2^{ab}	27
Isosaline	4	726^{ab}	0.72^{bcd}	84^{ab}	3.4^{a}	0.22^{ab}	27
Hyposaline	Iso N	593^{bc}	0.78^{abc}	73^{c}	2.7^{ab}	0.09^{b}	24
Isosaline	Iso N	518^{bc}	0.66^{cd}	89^{a}	1.99^{b}	0.13^{ab}	26
Hyposaline	1 Iso N	859^{a}	0.92^{a}	64^{d}	2.51^{ab}	0.23^{ab}	26
Isosaline	1 Iso N	726^{ab}	0.75^{bc}	78^{bc}	3.06^{a}	0.31^{ab}	28
Hyposaline	2 Iso N	639^{abc}	0.83^{ab}	64^{d}	2.87^{ab}	0.35^{ab}	26
Isosaline	2 Iso N	631^{abc}	0.73^{bcd}	78^{bc}	2.98^{ab}	0.31^{ab}	26
			Means of main effects				
Salinity							
Hyposaline		653.6	0.82*	71.1	2.85	0.21	26.1
Isosaline		617	0.70	81.6*	2.84	0.27	26.9
	Basal	481.5^{d}	87.5	0.655^{b}	2.75^{ab}	0.19^{ab}	26^{ab}
	1	572.5^{cd}	75.5	0.775^{ab}	2.8^{ab}	0.242^{ab}	26.5^{ab}
	2	692.5^{abc}	70	0.785^{ab}	3.04^{ab}	0.34^{a}	28^{a}
	4	717.5^{ab}	78.5	0.79^{ab}	3.265^{a}	0.21^{ab}	27^{ab}
	Basal Iso N	555.5^{cd}	81	0.72^{ab}	2.345^{b}	0.11^{b}	25^{b}
	1 Iso N	792.5^{a}	71	0.835^{a}	2.785^{ab}	0.27^{ab}	27^{ab}
	2 Iso N	635^{bc}	78	0.71^{ab}	2.925^{ab}	0.33^{ab}	26^{ab}
			2-Factor ANOVA: P-values				
salinity		0.346	0.0246	0.0001	0.9768	0.1379	0.0843
creatine		0.0001	0.3911	0.0231	0.0123	0.0374	0.0335
salinity x creatine		0.7142	0.6171	0.8405	0.0717	0.286	0.6704

[1]Initial weight was 1.4± 0.6g fish. There were no significant differences among treatments (ANOVA: P > 0.05).

Table 26. Proximate composition of whole-body of juvenile red drum fed graded levels of creatine in hypo- and isosaline environments for 8 weeks.

Variable	Creatine %	Dry Matter	Crude Protein	Crude Lipid	Ash
		Individual treatment			
Salinity					
Hyposaline	Basal	28.2	16.9	6.7	3.6
Isosaline	Basal	25.9	17	6.8	3.4
Hyposaline	1	28.1	17.5	6.7	3.5
Isosaline	1	27.3	16.4	7.2	3.6
Hyposaline	2	26.5	16.5	7	3.4
Isosaline	2	28	17.6	7.1	3.7
Hyposaline	4	25.98	16.9	7	3.3
Isosaline	4	25.9	17.1	6.9	3.3
Hyposaline	Iso N	26.4	17.2	6.9	3.7
Isosaline	Iso N	27.2	16.8	6.9	3.5
Hyposaline	1 Iso N	26.9	16.9	6.5	3.5
Isosaline	1 Iso N	26.5	16.7	7.3	3.5
Hyposaline	2 Iso N	27.5	17.4	6.7	3.4
Isosaline	2 Iso N	26.4	17	7.3	3.5
		Means of main effects			
Salinity					
Hyposaline		27.1	17	6.8	3.5
Isosaline		26.7	17	7.1	3.5
	Basal	27.1	17	6.8	3.5
	1	27.7	17	7	3.6
	2	27.3	17.1	7.1	3.6
	4	25.9	17.0	7	3.3
	Iso N	26.8	17.0	6.9	3.6
	1 Iso N	26.7	16.8	6.9	3.5
	2 Iso N	27	17.2	7	3.5
		2-Factor ANOVA: P-values			
salinity		0.12	0.38	0.07	0.14
creatine		0.56	0.76	0.23	0.46
salinity x creatine		0.68	0.89	0.55	0.36

Table 27. Proximate composition of muscle of juvenile red drum fed graded levels of creatine in hypo- water and isosaline environments for 8 weeks.

Variable	Creatine %	Dry matter	Protein	Lipid	Creatine
		Individual treatment			
Salinity					
Hyposaline	0	25.8	24.6	1.11	0.31^{c}
Isosaline	0	25.7	23.9	1.08	0.35^{bc}
Hyposaline	1	26.3	25.4	1.14	0.37^{b}
Isosaline	1	25.6	24.6	1.19	0.39^{ab}
Hyposaline	2	26.8	25.7	1.01	0.4^{ab}
Isosaline	2	26.5	26.4	1.13	0.44^{a}
Hyposaline	4	25.8	25.3	1.11	0.41^{a}
Isosaline	4	26.3	24.8	1.15	0.46^{a}
Hyposaline	Basal Iso N	25.9	24.2	0.99	0.35^{bc}
Isosaline	Iso N	25.6	23.8	1.21	0.36^{bc}
Hyposaline	1 Iso N	26.9	24.1	1.2	0.41^{a}
Isosaline	1 Iso N	27.1	25.5	1.24	0.42^{a}
Hyposaline	2 Iso N	26.6	25.6	1.3	0.4^{ab}
Isosaline	2 Iso N	25.6	24.7	1.17	0.38^{bc}
		Means of main effects			
Salinity					
Hyposaline		26.3	25	1.1	0.38
Isosaline		26.1	24.8	1.1	0.4
	0	25.8	24.3	1.1	0.33^{b}
	1	26	25	1.2	0.38^{ab}
	2	26.7	26.1	1.1	0.42^{a}
	4	26.1	25.1	1.1	0.435^{a}
	Iso N	25.8	24	1.1	0.355^{b}
	1 Iso N	27	24.8	1.2	0.415^{a}
	2 Iso N	26.1	25.2	1.2	0.39^{ab}
		2-Factor ANOVA: P-values			
Salinity		0.28	0.18	0.73	0.13
Creatine		0.06	0.62	0.34	0.0001
Salinity x Creatine		0.75	0.91	0.88	0.37

VI.4 Discussion

In hyposmotic and isosmotic environments, creatine supplementation statistically improved weight gain of red drum as previously demonstrated (Burns and Gatlin, 2016). Though there was no evidence of an interaction of salinity and diet in this trial (6 ppt to 11 ppt), a more dramatic salinity difference than seen in the current study should be considered. Isosmotic salinity in red drum was previously determined to be approximately 11 ppt (Weirich and Tomasso 1991), and though osmoregulation is energy consuming (Bryan *et al.* 1988), optimal growth for red drum has generally been detected at lower salinities (Brett 1979, Tomasso and Kempton 2000). Previously, I detected improved growth of red drum in hyposmotic (5 ppt) and hyperosmotic (15 ppt) conditions with creatine supplementation in trial I and II respectively. This study confirms that red drum performance is improved in isosmotic waters as well. Recently, (Schrama *et al.* 2018) found gilthead seabream raised in full strength seawater showed no improvement in growth performance with creatine supplementation after 69 days, and (McFarlane *et al.* 2001) found no growth improvements in rainbow trout after 7 days in freshwater. Discrepancy regarding the impact of creatine supplementation on growth of fishes is likely to be species specific.

Survival and feed efficiency of red drum in the present study tended to be inversely correlated to water salinity. However, previous studies do not suggest that lower salinity negatively affects survival of juvenile red drum (Gatlin *et al.* 1992, Craig *et al.* 1995), therefore, differences in survival in this study is unclear.

Full strength seawater was shown to increase HSI of red drum when compared to fish reared in fresh or brackish water, but not when comparing fish from fresh vs brackish water

(Craig *et al.* 1995). Results in this study is consistent with those. Furthermore, dietary creatine increased HSI as I previously observed in trial I and II.

Muscle creatine levels of red drum ranged from 0.31 to 0.46 representing a baseline and saturation. There is potential for diets with greater than 4% creatine inclusion to more rapidly increase muscle creatine concentration. Excessive creatine showed no deleterious effects on red drum in the current study. In humans, creatine is supplemented in a loading phase, where individuals consume 20 g/day for 6 days, and a maintenance phase where individuals continuously consume 2 g/day (Hultman *et al.* 1996). During the loading phase, muscle total creatine concentration increases by 20% and is maintained during the maintenance phase (Hultman *et al.* 1996). Supplementation during the loading phase, for an average human male, is approximately 0.3 g/kg/day. In the current study, red drum consumed between 0.6 g/kg/day and 2.4 g/kg/day of creatine based on their initial weight. Creatine-supplemented diets improved total muscle creatine of red drum by approximately 19%. Similar levels of creatine where fed to sea bream without increasing total muscle creatine (Schrama *et al.* 2018). Muscle creatine in rainbow trout did not increase after 7 days of supplementation, but did increase muscle glycogen and improve sprint performance (McFarlane *et al.* 2001).

Supplemental arginine and glycine did not increase muscle creatine levels in the present study. Early evidence suggests enzymes for creatine biosynthesis are located in muscle tissue of fish (Borchel *et al.* 2014) as opposed to the liver and kidney in other vertebrates (Joncquel-Chevalier Curt *et al.* 2015). Thus, excess free dietary arginine is likely purposed for other metabolic functions such as nitric oxide production (Buentello and Gatlin 1999). Arginine supplementation in red drum above the minimum dietary requirement has shown slight

improvements in growth performance (Cheng *et al.* 2011), and the results in this study are consistent

Conclusions

This study concludes that creatine improves the growth performance of red drum in low salinity and moderate salinity environments. Whether salinity affects dietary creatine requirements of red drum needs further examination, but at 5 ppt, optimal supplementation was quantified at 19.8 g/kg (1.98% of diet). Excess creatine of 37.8 g/kg (3.78% of diet) did not result in any deleterious effects on red drum.

CHAPTER VII

CONCLUSIONS

Aquaculture is the fastest growing agriculture in the world and must continue to grow to keep up with demands from an increasing human population. The expansion of aquaculture depends on the ability of researchers and culturist to efficiently produce fishes with minium input to achieve sustainability. The collection of studies in this dissertation demonstrates the vastness of fish physiology, behavior, and nutrition as each species adapts to environmental changes differently.

Exercise by forced swimming had a wide range of impacts on organ and body composition of the fish species evaluated. Seemingly, exercise is beneficial to most animals; fishes included. Though hybrid striped bass (HSB) have a natural history more akin to salmonids, I found that system construction and feeding method negatively impacted their feeding behavior, while red drum and tilapia positively took to their environment and actively fed. Evaluating effects of fish exercise on non-salmonid species is difficult when inconsistencies in research methods differ and fishes uniquely react to these methods. Yet, general consensus suggest, as I would conclude, that aerobic exercise, at low speeds, is beneficial to the health and growth of fishes.

Creatine is one of the most researched natural supplements (Cooper *et al.* 2012), yet, evaluation of the supplementation of creatine in the diets of fishes is sparse. Creatine is known to have ergogenic improvements on mammals, particularly during anaerobic exercise. Due to difficulty in system construction, anaerobic exercise of fishes has been seldom attempted. Furthermore, creatine is minimumly included in practicle diets, and expensive to add to

formulated diets. For these reasons, research on dietary creatine supplementation in fish is lacking.

Alternatively, there is no question fishes endogenously produce and consume creatine. The impact of dietary creatine in red drum suggest a broad range of potential impact that should be extensively researched; in particularly improved robustness. For catfish and HSB, benefits were more precise as creatine assisted osmoregulation in unfavorable saline environments. Without the added osmoregulatory stressor of salinity, creatine showed no improvements in growth performance. Furthermore, the study with HSB suggests circulating creatine and not stored creatine impacts osmoregulation. Though creatine is an expensive supplement, addition of creatine during abrupt osmotic changes can be beneficial to the health and survival of fishes.

Finally, this dissertation concludes that there is a mechanism for exogenous creatine to be absored by muscle tissue in fishes. This process is slow compared mammals and may be species specific.

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